MECHANISMS UNDERLYING CARDIOVASCULAR BENEFITS OF SODIUM GLUCOSE CO-TRANSPORTER-2 INHIBITORS: MYOCARDIAL SUBSTRATE OR SODIUM/HYDROGEN EXCHANGER?

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DEDICATION

This work is dedicated to my husband, Mike, and our daughters, Audrey and Margret. Without their love, help, patience, and encouragement, this would have never been possible.

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MECHANISMS UNDERLYING CARDIOVASCULAR BENEFITS OF SODIUM GLUCOSE CO-TRANSPORTER-2 INHIBITORS: MYOCARDIAL SUBSTRATE OR SODIUM/HYDROGEN EXCHANGER?

Recent clinical outcome studies demonstrate that Sodium glucose cotransporter 2 inhibitors (SGLT2i) significantly reduce major adverse cardiovascular events and heart failure outcomes in subjects with type 2 diabetes mellitus. At present, several hypotheses have been proposed to explain the observed cardiovascular benefit of SGLT2i, however, the mechanisms responsible remain to be elucidated. This investigation tested the hypothesis that SGLT2i improves cardiac function and efficiency during acute, regional ischemia/reperfusion injury via preferential shifts in myocardial substrate selection and/or inhibition of cardiac sodium/hydrogen exchanger-1 (NHE-1).

Our initial investigation evaluated the effects of 24 hour pretreatment of the SGLT2i canagliflozin on cardiac contractile function, substrate utilization, and efficiency before and during regional myocardial ischemia/reperfusion injury in healthy swine. At the onset of ischemia, canagliflozin increased left ventricular end diastolic and systolic volumes which returned to baseline with reperfusion. This increased end diastolic volume was directly associated with increased stroke volume and stroke work relative to controls during ischemia. Canagliflozin also increased cardiac work efficiency during ischemia relative to control swine. No differences in myocardial substrate uptake of glucose, lactate, fatty acids or ketones were detected between groups. In separate experiments using a longer 60 min coronary occlusion, canagliflozin significantly diminished myocardial infarct size.

Subsequent studies investigated the effect of an acute administration (15-30 min pre-treatment) of canagliflozin and the NHE-1i cariporide on cardiac contractile function

efficiency in response to myocardial ischemia/reperfusion injury. Similar to our initial studies, canagliflozin increased diastolic filling, stroke work and improved cardiac work efficiency relative to untreated control hearts during the ischemic period. In contrast, cariporide did not alter ventricular filling volume, cardiac output or work efficiency at any time point. Additional examination of AP-1 cells transfected with wild-type NHE-1 showed dose-dependent inhibition of NHE-1 activity by cariporide, while canagliflozin had minimal effect on overall activity. This investigation demonstrates that SGLT2i improves cardiac function and efficiency during acute, regional ischemia in healthy swine. However, the present data fail to support the hypothesis that these SGLT2i-mediated improvements involve either preferential alterations in myocardial substrate utilization or the inhibition of NHE-1 activity.

Johnathan Tune, PhD, Chair

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3-OHB -- Ketone body 3-hydroxybutyrate

AAR -- Area at risk

AP-1 cell -- antiporter-deficient cells

ATP -- Adenosine triphosphate

CHO -- Chinese hamster ovary

DM -- Diabetes mellitus

dP/dT -- change in pressure of time - a measure of load dependent cardiac contractile function

FDA -- Food and Drug Administration

FFA -- Free Fatty Acids

HCT -- Hematocrit

HFpEF -- Heart failure with preserved ejection fraction

I/R -- Ischemia/Reperfusion

iPSC -- Induced pluripotent stem cell

LAD -- Left anterior descending artery

LCX -- Left circumflex artery

MetS -- metabolic syndrome

MVO2 -- Myocardial oxygen consumption

NHE-1 -- Sodium hydrogen exchanger-1

NHE-1i -- Sodium hydrogen exchanger-1 inhibitor

PTM -- Post translational modification

ROR -- Rate of recovery

SGLT -- Sodium glucose cotransporter

SGLT2 -- Sodium glucose cotransporter-2

SGLT2i -- Sodium glucose cotransporter-2 inhibitor

TAC/MI -- transverse aortic constriction/myocardial infarction

Tau -- Left ventricle relaxation time constant

TTC -- 2, 3,5-triphenyltetrazolium chloride

WT -- Wild Type

Chapter 1: Introduction

Population at risk for cardiovascular disease

Cardiovascular disease is a group of diseases that affect the heart and/or blood vessels, including coronary heart disease, hypertensive cardiovascular disease, peripheral vascular disease, rheumatic heart disease, congenital heart disease. Constituting a considerable health care burden across the world, cardiovascular disease accounts for over 17 million deaths annually, and is a global concern killing more people than all cancers combined (Figure 1.1) [24, 127]. Cardiovascular disease is also the leading cause of death in the United States, with approximately 630,000 Americans dying from some form of heart disease each year (Figure 1.2) [122]. The American Heart Association estimates that almost half of the US population (>130 million people) will have some form of heart disease by 2035, with the estimated total medical costs (direct and indirect) for caring for these individuals reaching over $1 trillion in that same time frame. Significant contributors to the rising cardiovascular disease burden, as well as major cardiovascular risk factors, include increased prevalence of kidney disease and the continued epidemic of obesity, and the rising prevalence of type 2 diabetes. Cardiovascular disease represents a complex group of diseases, and unsurprisingly, is associated with numerous risk factors (e.g. smoking, high cholesterol, hypertension, obesity and diabetes). Although advances in the treatment of cardiovascular disease have been made, a better understanding of the complex interplay between cardiovascular risk factors is needed for further progress in this area.

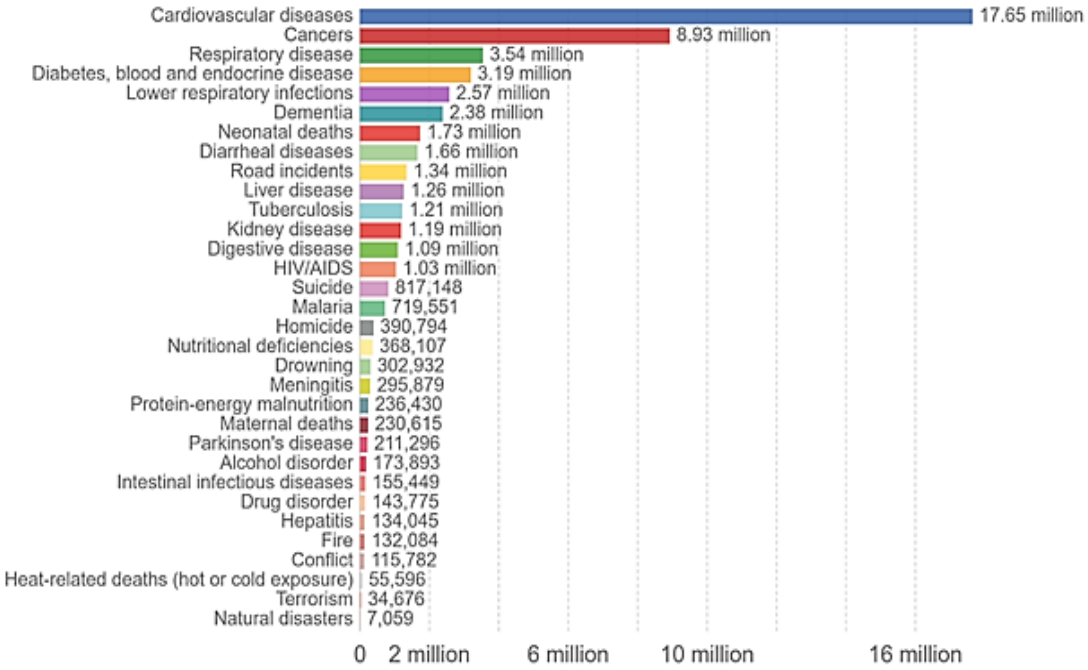


Figure 1.1 Number of deaths in 2016 by cause.
Specific cause of death globally regardless of risk factors (environmental, diet and other lifestyle factors. [24]

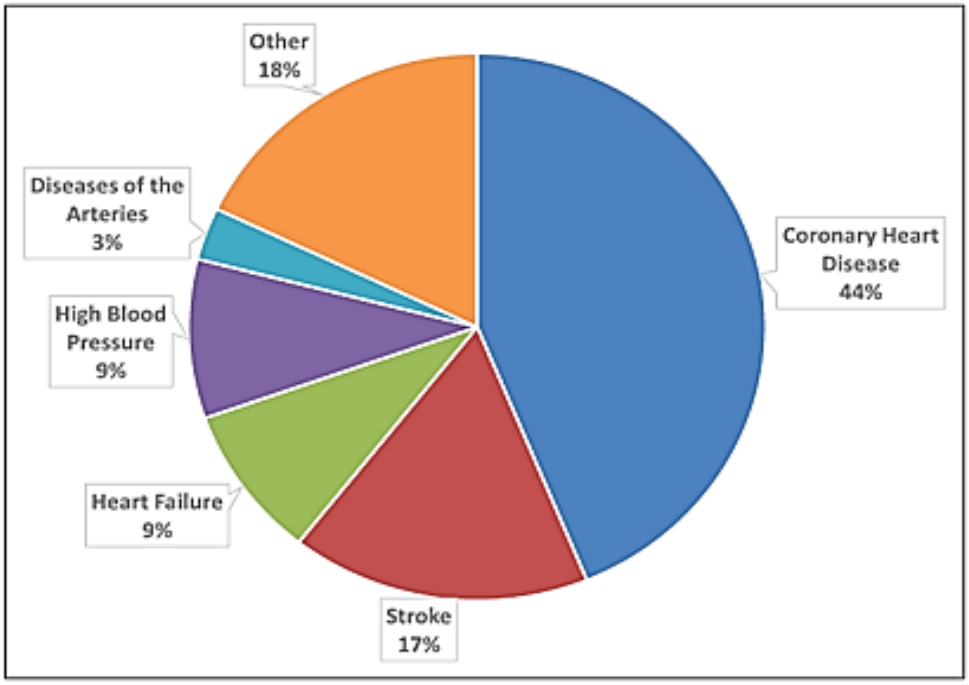


Figure 1.2 Breakdown of cardiovascular disease related deaths in the United States (2015). [24]

Cardiovascular disease in obesity and diabetes

Obesity, as defined by body mass index (BMI) ≥ 30 kg/m^2, makes up one of the most important cardiovascular disease risk factors as the prevalence of obesity has increased over the last several decades affecting approximately 2.1 billion adults globally (Figure 1.4) [87, 145]. Recent studies have estimated that 70% of adults in the United States are overweight or obese and that this is similar between men and women regardless of race/ethnicity (Figure 1.3) [44, 148]. Obesity, a chronic metabolic disease has a significant impact on the cardiovascular system. Obese men and women when compared to normal weight, gender and age matched individuals were more likely to have a stroke, heart attack, heart failure or cardiovascular death [87]. Obesity leads to several structural and functional changes in the cardiovascular system, including reduced cardiac output, increased systemic resistance, increased left ventricular mass and wall thickness and left ventricular systolic dysfunction [26].

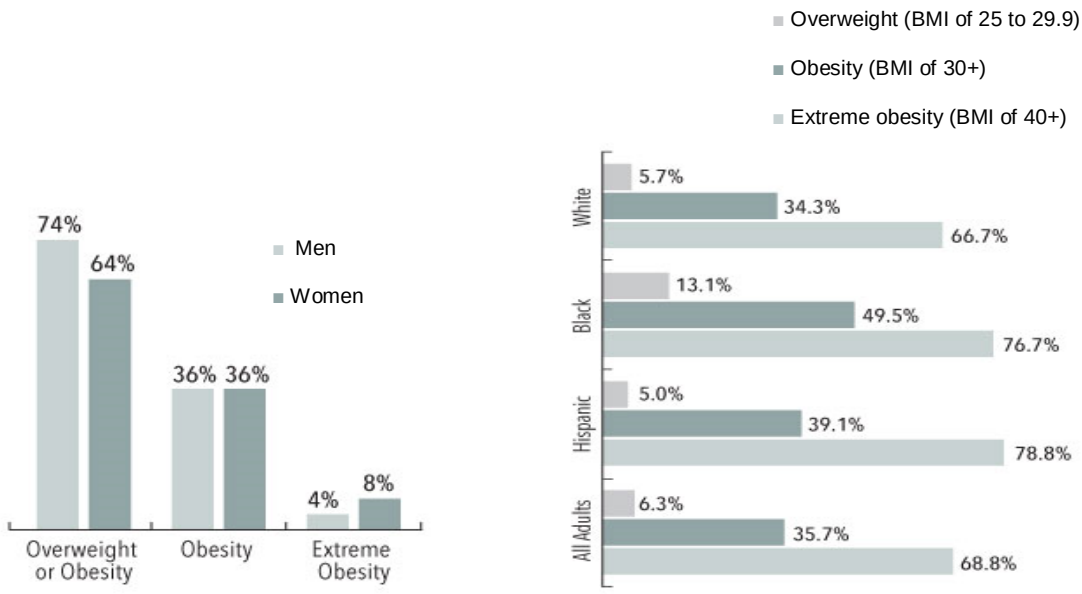


Figure 1.3 Incidence of obesity.
Estimate of overweight or obese adults in the United States. [44]

Obesity itself is associated with several cardiovascular risk factors including, insulin resistance and type 2 diabetes [6, 24, 38, 82, 148]. Approximately 90-95% of all diagnosed diabetic cases in adults are obesity related type 2 diabetes [135]. The prevalence of diabetes has also increased over the last several decades (Figure 1.4) and it is predicted that the number of diabetic individuals will increase by approximately 54% to over 54 million Americans by 2030. Suffering from type 1 or type 2 diabetes increases an individual's risk of a cardiovascular related death by up to 50%. Major causes of mortality in diabetic individuals includes, coronary heart disease, cerebrovascular disease and peripheral artery disease [137].

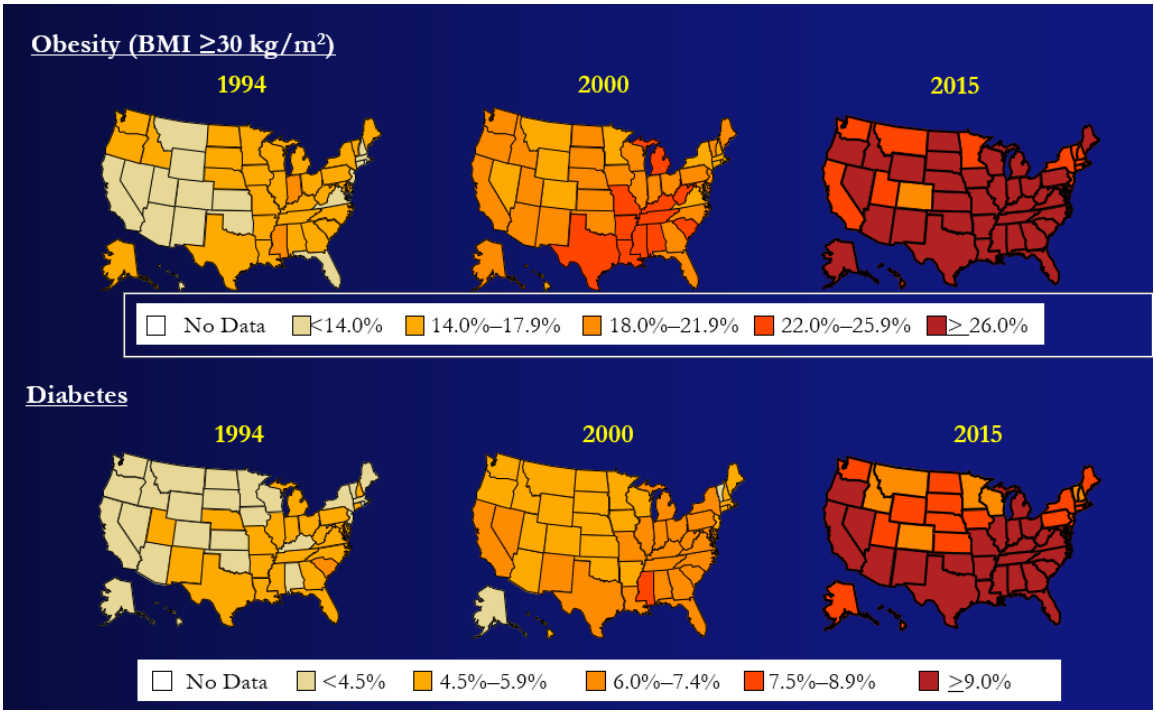


Figure 1.4 Age-adjusted prevalence of obesity and diabetes among adults in the United States. [122]

Obesity and diabetes are independent risk factors for cardiovascular disease and the combination of the two increases the rate and severity of cardiovascular disease [119, 124, 148]. Additionally, dysmetabolic individuals (i.e. obese individuals with insulin

resistance or type 2 diabetes) are associated with cardiovascular pathophysiological changes including changes in myocardial substrate metabolism, impaired oxygen supply/demand balance, concentric cardiac hypertrophy and diastolic contractile dysfunction [148]. These pathophysiological changes are distinct to this dysmetabolic population and the mechanisms driving these changes remain poorly understood. Moreover, many therapies used in type 2 diabetes management have failed to reduce adverse cardiovascular outcomes. Some therapies are even associated with an increased risk for incident heart failure in diabetic individuals [14, 51, 151]. Together this highlights the need to better understand the mechanisms driving the dysmetabolic cardiac changes and the need for new therapeutic options for treating cardiovascular disease in dysmetabolic individuals.

Anti-hyperglycemic therapies and cardiovascular risk

Numerous therapies are available to control glucose levels in diabetic individuals. However, despite having tightly controlled glucose levels, diabetic individuals still have an increased risk of heart disease and stroke. Metformin, sulfonylureas, meglitinides, thiazolindinediones, and dipeptidyl-peptidase-4 inhibitors,

despite their long term glucose control, all fail to reduce adverse cardiovascular outcomes [85]. The Action to Control Cardiovascular Risk in Diabetes (ACCORD) trial even suggested cardiovascular harm related to aggressive glucose lowering with anti-hyperglycemic agents [51, 57]. Additionally, in 2007 a meta-analysis published by Nissen and Wolski reported a 43% increase in myocardial infarction and a 64% increase in death from cardiovascular causes with rosiglitazone, an anti-diabetic drug in the thiazolidinedione class [73, 76]. Data from the RECORD (Rosiglitazone evaluated for cardiovascular outcomes in oral agent combination therapy for type 2 diabetes) trial demonstrated an increased risk for heart failure with rosiglitazone, no effect on

cardiovascular mortality and was inconclusive regarding myocardial infarction [75]. However, the alarming findings from the initial 2007 meta-analysis by Nissen and Wolski led the Food and Drug Administration (FDA) to issue a guidance in 2008 requiring cardiovascular disease risk assessment for all new glucose lowering drugs. In short, the new FDA guidance for industry requires pharmaceutical companies to demonstrate that the upper bound of the two-sided 95 percent confidence interval for the estimated hazard risk ratio for cardiovascular events including cardiovascular mortality, myocardial infarction, and stroke to be less than 1.3 (hazard ratio = treatment hazard rate/placebo hazard rate therefore, a parameter or outcome with a hazard ratio < 1 improves with treatment, >1 worsens with treatment) (Figure 1.5) [4, 76].

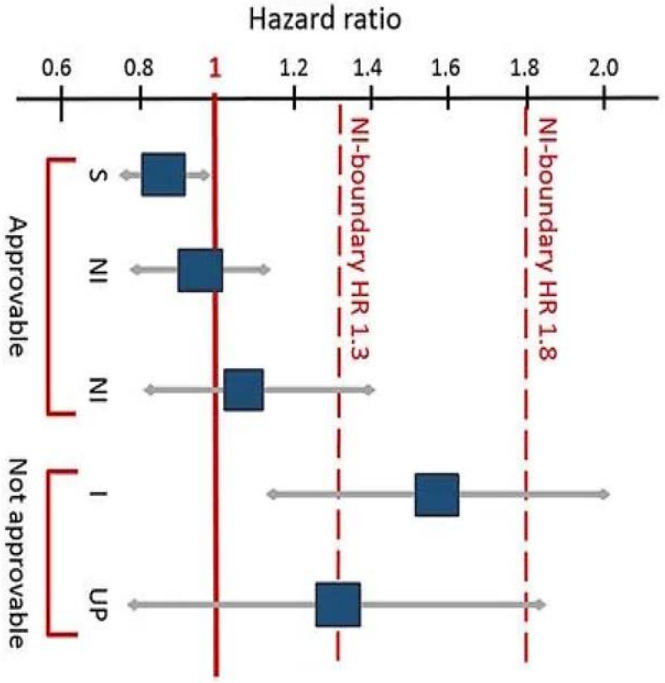


Figure 1.5 FDA guideline for glucose lowering drugs.
Examples of hazard ratios (HR) and the upper limit of the 95 % confidence interval (CI) and regulatory consequences of each outcome. S superiority, NI non-inferiority, I inferiority, UP underpowered. [4]

Since the FDA guidance was issued, two classes of glucose lowering drugs, dipeptyidyl-peptidase-4 inhibitors (DPP-4i), and glucagon-like peptide-1 receptor agonist (GLP-1 RA) have completed cardiovascular outcome trials. Three trials have reported outcome data for DPP-4i, SAVOR-TIMI for Saxagliptin, EXAMINE for alogliptin and TECOS for sitagliptin. All three trials met the primary outcome of less than 1.3 hazard ratio (HR) but, none were associated with any cardiovascular benefit [60, 139, 157]. Additionally, saxagliptin was associated with an increased risk for incident heart failure (HR 1.27) and the FDA recommended a warning label for aloglipitin use in patients with a history of heart and kidney disease [139, 157]. See figure 1.6.

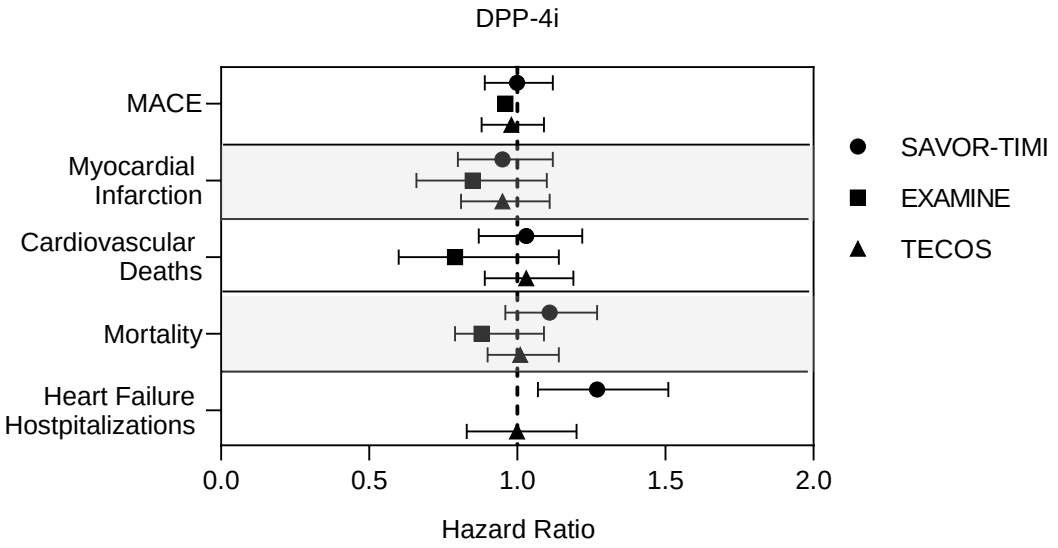


Figure 1.6 Cardiovascular outcomes for SAVOR-TIMI, EXAMINE AND TECOS TRIALS. [60, 139, 155]

There are currently 4 completed trials for GLP-1 RA. ELIXA, the first reported trial, demonstrated noninferiority of lixisenatide using a 4-point major adverse cardiovascular events (MACE) (composite of nonfatal stroke, nonfatal myocardial infarction, cardiovascular death and hospitalization for unstable angina), but did not find any cardiovascular benefit [121]. The LEADER trial which evaluated liraglutide treatment,

demonstrated a significant reduction in 3-point MACE (composite of nonfatal stroke, nonfatal myocardial infarction, and cardiovascular death) [106]. The benefit observed in the LEADER trial was mainly driven by the significant reductions in cardiovascular deaths. These findings led to a new indication for liraglutide, to reduce the risk of MACE in type 2 diabetic adults with cardiovascular disease [76]. SUSTAIN-6 confirmed noninferiority of long-acting semaglutide on 3-point MACE. Significant reductions in nonfatal stroke and trends towards decreased non-fatal myocardial infarctions were reported with semaglutide treatment [106]. The fourth reported trial, EXSCEL evaluated extended release exenatide. It confirmed noninferiority with no significant improvements in cardiovascular outcomes [74]. See figure 1.7.

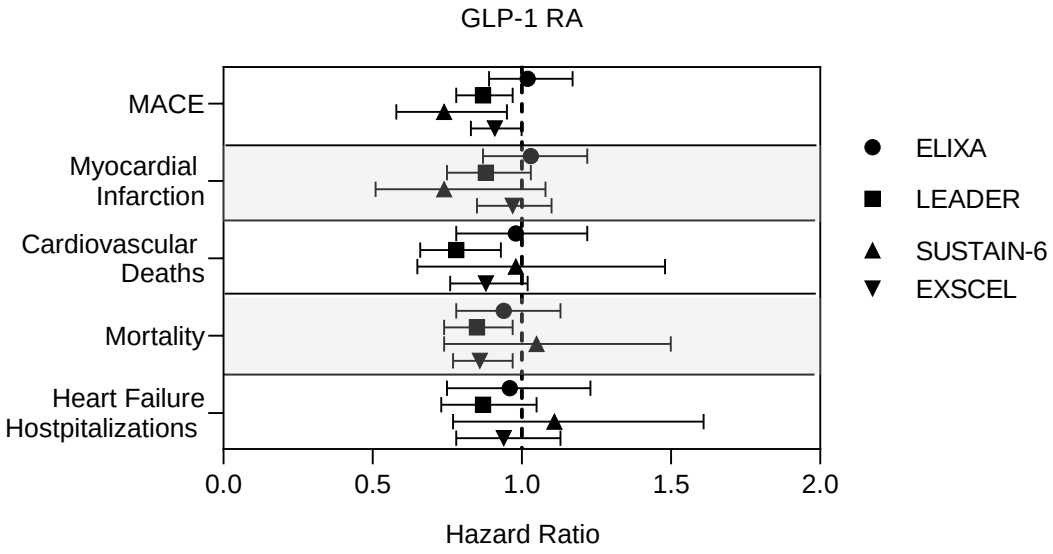


Figure 1.7 Cardiovascular outcomes for ELIXA, LEADER, SUSTAIN-6 AND EXSCEL. [74, 76, 106]

Since the 2008 FDA guidelines were issued several cardiovascular outcome trials have been completed and results published. These trials demonstrate a range of results, i.e. several agents were not associated with an increased cardiovascular risk (beyond an unacceptable level, HR > 1.3), some were associated with an increased risk for heart

failure (saxagliptin and alogliptin) and some agents (i.e. GLP-1 inhibitors, specifically liraglutide and semaglutide) demonstrated cardiovascular benefits. Together these data and the increased cardiovascular risk in dysmetabolic patients highlight the need for evaluating the appropriate glucose lowering treatment for type 2 diabetic patients at risk for cardiovascular disease as well as the need for new anti-hyperglycemic agents with added cardiovascular benefits.

Sodium glucose Transporter 2

In 1960, Bob Crane proposed the sodium/glucose cotransport hypothesis, that glucose was transported across the plasma membrane of the digestive surface (brush border of the intestines) by a sodium/glucose carrier complex (Figure 1.8). Crane believed this active transport of glucose was the result of the sodium gradient maintained by a second sodium pump (possibly the Na+/K pump) [159]. Prior to this there were no explanations for active membrane transport of glucose or other molecules in the body. Aside from some minor points, this model remains valid today [159].

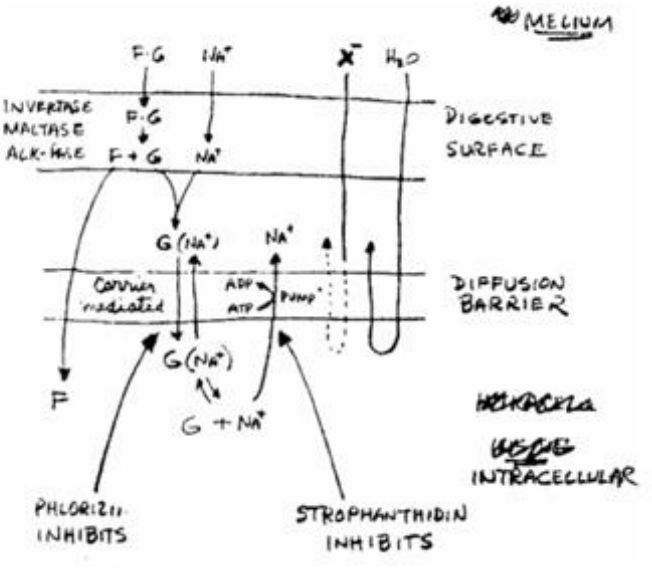


Figure 1.8 SGLT model drawn by Bob Crane. [159]

In the early 1980's researchers found that glucose transport in the proximal tubule of the kidneys was more rapid than in the distal tubule, this ultimately led to the discovery of sodium glucose cotransporters (SGLT) [159]. In 1987 Wright et al. successfully cloned

the intestinal sodium/glucose transporter SGLT1 in small intestines and in 1992 Wells et al. successfully identified and cloned SGLT2 in the kidney [156, 159].

Sodium glucose transporters are encoded by genes in the SLC5A family. The SGLT family consists of 6 members (SGLT1-6), SGLT1 and 2 are sodium dependent glucose cotransporters, SGLT3 a glucose sensor, SGLT4 and 6 are sodium dependent multivitamin transporters and SGLT5 a thyroid iodide transporter [134]. For this proposal, the focus will be on SGLT2 and briefly on SGLT1. SGLT2 is highly expressed in the kidney and in contrast to SGLT1 has a low affinity for glucose but, a high capacity for its transport with a 1:1 sodium to glucose coupling ratio (Figure 1.9) [134]. SGLT2 is in the S1 and S2 segments of the proximal tubules in the kidney and under normal conditions is responsible for 90% of glucose reabsorption. SGLT1 is in the S2 and S3 segments of the proximal tubule and is responsible for 10% of glucose reabsorption [31] (Figure 1.10). In hyperglycemia SGLT2 activity is upregulated ultimately contributing to hyperglycemia [107]. SGLT2 inhibitors (SGLT2i) block reabsorption of filtered glucose in the kidney resulting in glucose excretion in the urine leading to reduced plasma glucose levels. Because of their role in glucose reabsorption, SGLT, particularly SGLT2 are an ideal therapeutic target for insulin independent glucose lowering. In 2013 canagliflozin

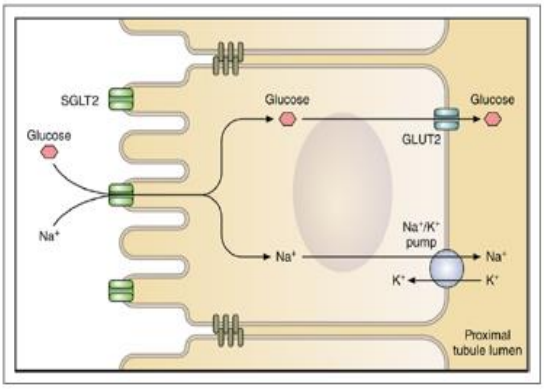


Figure 1.9 SGLT mechanism in the proximal tubule. [70]

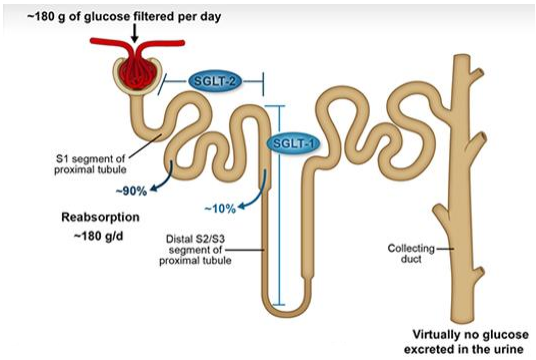


Figure 1.10 SGLT in the kidney and effect on glucose homeostasis. [31]

was the first FDA approved SGLT2i for treatment of type 2 diabetes. This was later followed by the approval of dapagliflozin and empagliflozin [152].

SGLT2i and cardiovascular events

FDA required studies of effects of SGLT2i on cardiovascular disease have yielded promising results. Three trials have been completed. The first was the EMPAREG OUTCOME trial evaluating empagliflozin treatment in T2DM patients with high cardiovascular risk. Empagliflozin significantly reduced 3-point MACE which was driven by a significant reduction in cardiovascular deaths [164]. Treatment with empagliflozin also resulted in significant reductions in all-cause mortality and heart failure hospitalizations [164]. The CANVAS trial was the second trial to be completed in patients with high cardiovascular risk. CANVAS, which evaluated the treatment effects of canagliflozin on 3-point MACE, also demonstrated significant reductions [114]. CANVAS reported reductions in mortality and heart failure hospitalizations although the differences were not significant [114]. DECLARE-TIMI 58, the most recently completed trial, confirmed noninferiority of dapagliflozin with no reductions in MACE in patients at risk for atherosclerotic cardiovascular disease [158]. Although dapagliflozin failed to reduce MACE, reductions in cardiovascular deaths and heart failure hospitalizations were reported [158]. See figure 1.11.

The most impressive results from these trials were the effects on heart failure hospitalizations and overall cardiovascular deaths. SGLT2i were the first glucose lowering drug class to be associated with significant reductions heart failure hospitalizations. Furthermore, the reduction in heart failure outcomes was observed within weeks of beginning treatment and was maintained for several years [164]. Additionally, all three SGLT2i have been found to significantly reduce cardiovascular mortality in type 2 diabetic patients. Data reported from the three SGLT2i trials were unexpected and together

indicate the improvements in cardiovascular outcomes with SGLT2i may be a class effect that warrants further investigation. See figure 1.12.

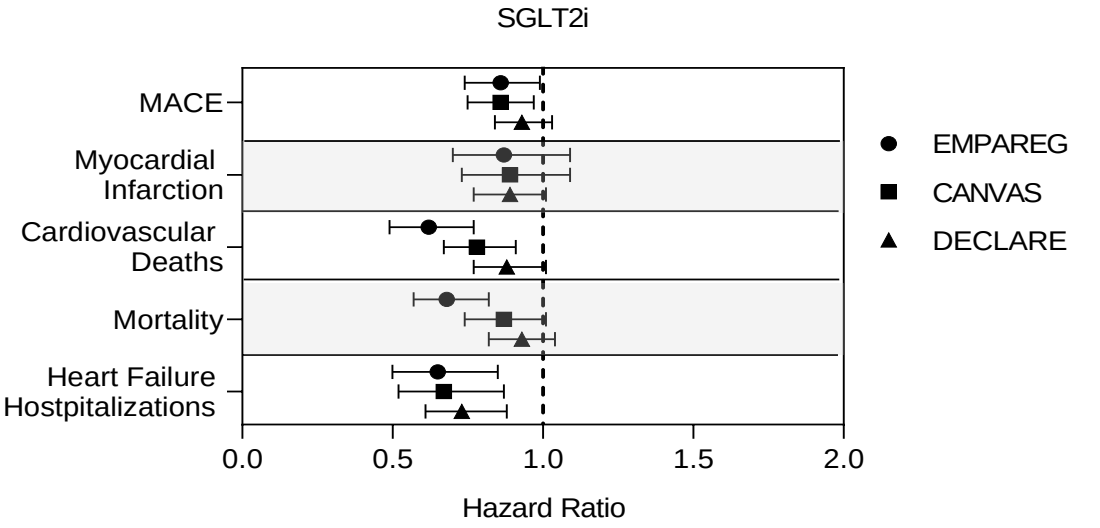


Figure 1.11 Cardiovascular outcomes for EMPAREG, CANVAS AND DECLARE. [114, 158, 164]

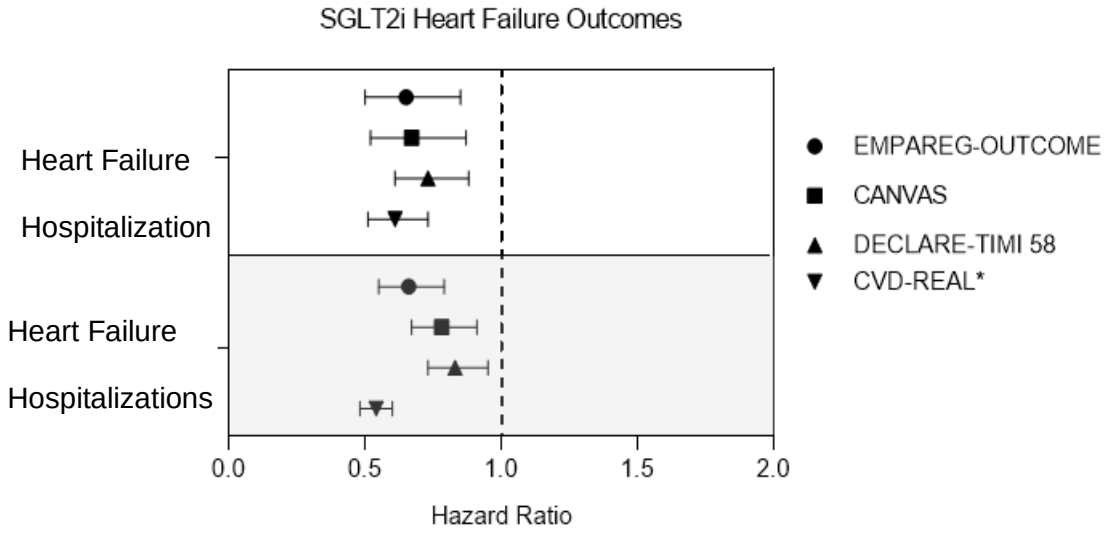


Figure 1.12 Heart failure outcomes for EMPAREG, CANVAS, DECLARE and CVD-REAL. [114, 158, 164]

SGLT2 expression

The cardiovascular benefits of SGLT2i are surprising and do not appear to be mediated by direct effects of these agents on the SGLT2 transporter in cardiovascular tissues, as SGLT2 protein expression is not detected in the heart or vasculature [32, 65, 68]. Zhou et al. performed early studies to identify SGLT1 and SGLT2 gene expression in 23 human tissues using real time polymerase chain reaction (RT-PCR). They reported high levels of SGLT1 expression in the small intestines and expression to varying degrees in the left ventricle, kidney, colon, testis, trachea, prostate, lung and liver. Zhou et al. also reported that SGLT2 mRNA was ubiquitously expressed in several human tissues with the highest level of expression in the kidney. This expression pattern of SGLT2 could be problematic as using an agonist or antagonist to a target found in a wide variety of tissues could cause unanticipated, unwanted effects. Following the studies of Zhou et al., Chen et al. set out to extensively evaluate the mRNA expression profile of SGLT2 and several of its related family members (SGLT1, SAAT1 (also known as SGLT3), SMIT (sodium myoinositol cotransporter 1), SGLT4, SGLT5 and SGLT6). These studies used quantitative RT-PCR to evaluate 72 normal human tissues from three separate donors. Additionally, they used five different primer/probe sets spanning the entire SGLT2 mRNA. Chen et al. found similar expression of SGLT1 as Zhou et al. however, the pattern of SGLT2 expression differed. Chen et al. concluded that the expression pattern of SGLT2 was highly restricted to the kidney in humans (Figure 1.13) and was not ubiquitously expressed as stated by Zhou et al. Further, additional groups have confirmed Chen el al's, findings that SGLT2 mRNA expression was only observed in the kidneys in humans and was not expressed in the heart [43, 153]. Due to the lack of SGLT2 expression in the heart, precisely how SGLT2i improves cardiovascular outcomes remains an enigma.

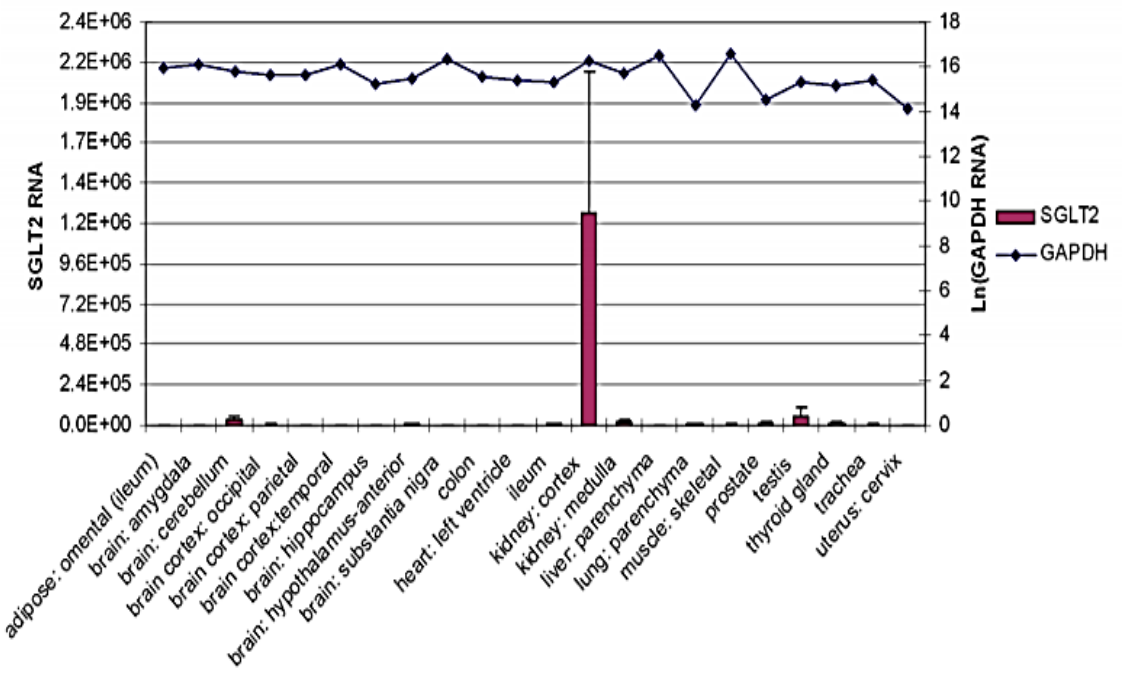


Figure 1.13 Quantitative PCR tissue expression profiling of thE SGLT2 gene in various human tissues**.** [32]

Proposed mechanisms of cardiovascular protection

Numerous mechanisms have been proposed for the observed SGLT2i cardiovascular benefits, including diminished artery stiffness, effects on sodium/fluid loss and weight loss, influences on renin-angiotensin system and anti-hypertensive effects, anti-inflammatory capacity and AMPK activation [33-35, 69, 92, 109, 150] (Figure 1.14). Additionally, several pre-clinical studies attempting to elucidate the proposed SGLT2i mediated cardioprotective mechanisms have been published (Table 1). These published pre-clinical studies demonstrate a variety of potential SGLT2i mediated effects (i.e. improved cardiac function, reduced atherosclerosis, modulation of neurohormones, reduced inflammation and fibrosis) [64, 94, 140, 162] in various pre-clinical models (i.e. Diabetic Akita mice, STZ-induced diabetic rats, ZDF diabetic and ZL nondiabetic rats, nondiabetic pigs) [63, 80, 97, 130]. While there is an abundance of data attempting to explain the SGLT2i mediated cardiovascular effects, some inconsistencies exist within the

published pre-clinical studies. For example, Chan et al., observed no improvements in blood glucose, systolic blood pressure (SBP), glomerular filtration rate (GFR) or urinary albumin creatinine ratio in the diabetic Akita mouse model when treated with canagliflozin [80]. Demarco et al. observed improvements in glycemia and proteinuria in obese/diabetic db/db mice with empagliflozin treatment [61] and Abu Seif et al. demonstrated blood pressure reductions in albino rats treated with canagliflozin [2]. Santos-Gallego et al. evaluated the effects of empagliflozin in nondiabetic pigs and demonstrated improved left ventricular function due to improved myocardial energetics [130] however, Lim et al. reported reduced myocardial infarct size in both diabetic and nondiabetic rats with canagliflozin treatment independent of glycemic status [97].

Several hypotheses have been proposed to explain the SGLT2i cardiovascular benefits and the literature appears to be settling on two, the “thrifty fuel hypothesis” and the “sodium hypothesis”. Recent published literature suggests effects on myocardial substrate metabolism, secondary to changes in systemic substrate availability, could explain the cardiovascular benefits of SGLT2i [1, 39, 46-48, 83, 123, 143]. This is referred to as the “thrifty fuel hypothesis”. Other evidence suggests that the SGLT2i mediated cardioprotective effects could be due to direct inhibition of the sodium hydrogen exchanger-1 (NHE-1). This is called the “sodium hypothesis” [19, 23, 25, 149].

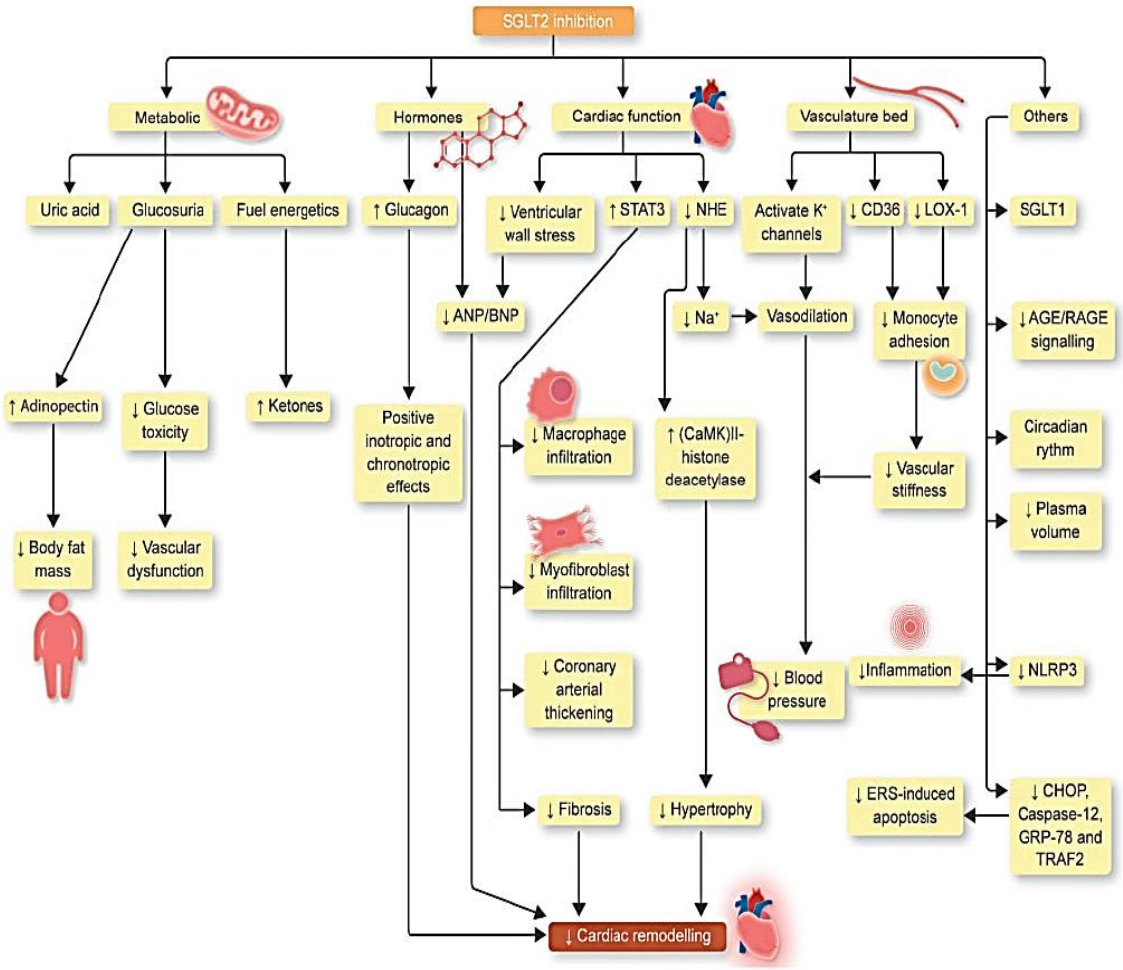


Figure 1.14: Potential mechanisms underlying cardiovascular benefits of SGLT2i. [35]

Table 1 SGLT2i pre-clinical studies

Mechanism	Outcomes	Animal model and SGLT2 inhibitor	Findings	Reference
Cardiac Function	• Dysglycemia, proteinuria, aortic stiffness, and diastolic dysfunction	• Obese/diabetic female db/db mice. • Oral empagliflozin (10 mg/kg/day) for 5 weeks	• Improvements in septal wall motion, flow propagation velocity, left ventricular filling pressure, isovolumic relaxation time and myocardial performance	Habibi et al., 2015 [61]
	• Cardiovascular injury and cognitive decline	• db/db mice • Standard diet or standard diet containing 0.03% empagliflozin for 10 weeks	• Ameliorated cardiovascular injury, remodeling, vascular dysfunction, and cognitive decline	Lin et al., 2014 [98]
	• Myocardial fibrosis through regulation of macrophage phenotypes and SGLT1 augmentation • Role of signal transducer and activator of transcription 3 (STAT3) in cardiac fibrosis	• in vivo and ex vivo experiments • Non-diabetic male Wistar rats • Dapagliflozin (0.1 mg/kg/day by oral gavage), phlorizin (0.4 g/kg/day injected s.q.), combination of dapagliflozin + S3I-201 (10 mg/kg per day, a STAT3 inhibitor, injected i.p.), or phlorizin + S3I-201	• Attenuated myofibroblast infiltration and cardiac fibrosis via increased STAT3 activity and STAT3 nuclear translocation	Lee et al., 2017 [95]
	• OGlcNAcylated protein levels	• Lipodystrophic Bscl2-/- (SKO) and wild-type (control) mice • Dapagliflozin (1 mg/kg in drinking water for 8 weeks)	• Reduced O-GlcNAcylated FOXO1 levels and prevented development of hypertrophic cardiomyopathy	Joubert et al., 2017 [81]
	• NLRP3 and inflammasome activation	• BTBR ob/ob and wild-type (control) mice • Dapagliflozin (1 mg/kg/day mixed with food) for 8 weeks	• Attenuated the development of fibrosis and remodeling and deterioration of left ventricular function via NLRP3, TNFa, and caspase-1	Ye et al., 2017 [162]

	• Cardiac function	• C57Bl/6 mice • Transverse aortic constriction • Empagliflozin (10mg/kg) daily by oral gavage for 2 weeks	• Increased cardiac output and cardiac work	Byrne, 2017 [29]
	• Myocardial function and injury	• Sprague-Dawley rats • ischemia/reperfusion (I/R) • Canagliflozin (3ug/kg)/IV/5th min of ischemia	• Reduced infarct size, increased efficiency during I/R	Sayour, 2019 [131]
	• Left ventricle function and injury	• DIO-insulin resistant rats for 4 weeks • ischemia/reperfusion (I/R) • Dapagliflozin (1mg/kg) daily by oral gavage for 4 weeks	• Reduced infarct size and maintained stroke work during I/R	Tanajak, 2018 [146]
	• Myocardial ischemia/reperfusion injury in diabetic and non-diabetic hearts	• Diabetic ZDF and non-diabetic ZL rats • high fat diet for 32 weeks • Canagliflozin (300 mg) daily orally for 4 weeks • Ischemia /reperfusion (IR)	• Attenuated myocardial infarct size independent of glycemic status	Lim, 2019 [97]
	• Cardiac fibrosis and ventricular hemodynamics	• Spontaneous Hypertensive Rats (SHR) • Empagliflozin (20 mg/kg) daily orally for 12 weeks	• Improved hemodynamics, attenuated cardiac fibrosis and normalized heart failure genes	Lee, 2019 [94]
	• Microvascular function and cardiac contractility	• Lean and ob/ob $^{-/-}$ mice • Empagliflozin (1.5 mg/kg) daily for 10 weeks	• Improved coronary flow reserve and fractional area change	Adingupu, 2019 [3]
	• Cardiac fibrosis and heart failure	• Non-diabetic pigs • 2-hour balloon occlusion • Empagliflozin	• Improved myocardial energetics, enhanced LV systolic function and ameliorated adverse LV remodeling	Santos-Gallego, 2019 [130]

		(10 mg) daily for 8 weeks		
NHE	• Cardiac NHE activity, hemodynamic and metabolic performance of isolated hearts tissues	• Molecular docking simulations • Langendorff-perfused mouse heart, perfused for 30 min • Empagliflozin (1 μM), dapagliflozin (1 μM), or canagliflozin (3 μM)	• All SGLT2i exhibit a class effect by blocking NHE and reducing $[Na^+]c$ directly in the cardiac cell. Canagliflozin and empagliflozin induce vasodilation of the coronary circulation of the intact heart, and empagliflozin increases oxygen consumption	Uthman et al., 2017 [149]
Atherosclerosis	• Repetitive fluctuations in blood glucose concentrations and monocyte adhesion to the aortic endothelium	• Male Goto-Kakizaki rats • Phlorizin (100 mg/kg injected subcutaneously twice daily) for 12 weeks	• Suppressed adhesion of monocytes on aortic endothelium	Azuma et al., 2006 [17]
	• Atherosclerosis	• Atherosclerosis model of male ApoE-/- mice • Empagliflozin 1 mg/kg and empagliflozin 3mg/kg for 8 weeks	• Decreased insulin resistance and circulating concentrations of TNF-alpha, IL-6, monocyte chemoattractant protein-1 (MCP-1), serum amyloid A and urinary microalbumin, and this significantly correlated with plaque size • Increased adiponectin levels	Han et al., 2017 [64]
	• Glycaemia and macrophage-driven atherosclerosis	• Glycaemia and macrophage-driven atherosclerosis • Apolipoprotein E null (ApoE-/-) mice, streptozotocin-induced diabetic ApoE-/- mice, and diabetic db/db mice • Dapagliflozin or ipragliflozin at the dose of 1.0	• Attenuated the up-regulation of gene expression of CD36 and Lox-1, surface areas of atherosclerotic lesions, atheromatous plaque size, macrophage infiltration, and foam cell formation	Terasaki et al., 2015 [147]

		mg/kg/day for 4 weeks		
Modulation of neurohormones	• Expression of biomarkers of heart failure and mortality	• AA-induced heart failure model in developing cmlc2::GFP transgenic zebrafish embryos • Empagliflozin (0.1, 10 lM)	• Modulated BNP and ANP signaling pathways, reduced the morphological and functional cardiac changes induced by AA, dampened AA-enhanced expression of BNP and ANP, and reduced embryonic mortality	Shi et al., 2017 [140]
Blood pressure	• Efficacy and safety of canagliflozin compared with vildagliptin	• Albino rats • Canagliflozin 10 mg/kg • n = 12	• Reduced mean blood pressure from baseline	Abu Seif et al., 2017 [2]
	• Regulation of vascular tone as well as NO-induced vascular relaxation	• Male streptozotocin-induced C57BL/6 diabetic mice • Canagliflozin (30 mg_10 mL-1_kg body weight-1 via oral gavage daily) or vehicle (0.5% HPMC) for 4 weeks. Mouse and human PAECs, human PASMCs, human CAECs, and human CASMCs were used in assays	• Augmented sodium nitroprusside-dependent relaxation in coronary arteries through induction of membrane hyperpolarization in smooth muscle cells, mediated by activation of potassium channels, but not in papaverine-induced model	Han et al., 2016 [65]
	• Blood glucose, SBP, GFR, urinary ACR, renal oxidative stress, angiotensinogen, and Nrf2 expression	• Adult male Akita mice • Canagliflozin (0.3 mg/mL in drinking water) for 16 weeks	• Improvements were not observed	Chan et al., 2016 [80]

Sympathetic nervous system	• Cardioprotective effects via glucagon, which has inotropic effects on the heart	• Male C57BL6 mice • Canagliflozin (10 mg/kg/day for 4 weeks)	• Reduced heart size, left ventricular dilation, and circulating glucagon concentration	Kamihara et al., 2017 [84]
	• Ventricular myocyte shortening and intracellular Ca^{2+} transport	• Male Wistar STZ-induced diabetic rats • Freshly isolated ventricular myocytes perfused with dapagliflozin (10-5 M, 10-6 M, and 10-7 M)	• Reduced the amplitude of ventricular myocytes shortening, calcium transient and L-type calcium current, suggesting that alteration in the mechanism(s) of calcium transport may partly explain the negative inotropic effects of SGLT2 inhibitors	Hamouda et al., 2015 [63]
Oxidative stress	• Endothelial dysfunction, oxidative stress, AGE/RAGE signaling and inflammation	• Endothelial dysfunction, oxidative stress, AGE/RAGE signaling and inflammation • Male Wistar rats • Empagliflozin (10 mg/kg/day oral) or empagliflozin (30 mg/kg/day oral) for 8 weeks	• Reduced blood glucose levels, normalized endothelial function and reduced oxidative stress in aortic vessels and in blood • Reversed the pro-inflammatory phenotype and glucotoxicity (AGE/RAGE signaling)	Oelze et al., 2014 [117]
Inflammation	• Activation of NLRP3 and inflammasome	• Male BTBR ob/ob and WT mice • Dapagliflozin (1 mg/kg/day) for 8 weeks	• Attenuated the activation of the nucleotide binding oligomerization domain (NOD)-like receptor 3 (NLRP3) inflammasome and upregulation of C-reactive protein. The anti-inflammatory effect was AMPK-dependent and SGLT1-independent	Ye et al., 2017 [162]
Endoplasmic reticulum stress	• Diabetic cardiomyopathy • Elucidate the related mechanism	• Male streptozotocin-induced Wistar rats • Empagliflozin (30 mg/kg/day oral); or empagliflozin	• Improved mean pressure–volume relationships, ventricular contractility, histopathologic changes and ERS-induced apoptosis of cardiomyocytes by	Zhou et al., 2017 [163]

		(10 mg/kg/day) for 8 weeks	downregulating proteins of the apoptosis pathways	

Thrifty fuel hypothesis

The “thrifty fuel hypothesis" [46, 111] proposes that the cardioprotective effects of SGLT2i are the result of a change in substrates utilized by the heart. The heart obtains the majority of its energy through mitochondrial oxidative phosphorylation [37]. Under normal conditions, the fuel source for the mitochondria is ~70% circulating free fatty acids and ~30% glucose [37, 101]. However, with myocardial ischemia or heart failure, and in the setting of diabetes, there is a preferential shift in fuel selection where the heart utilizes fatty acids almost exclusively [22, 101]. Oxidation of fatty acids requires more oxygen per unit of adenosine triphosphate (ATP) than other substrates (Figure 1.15) and an excess of fatty acid oxidation has been associated with reactive oxygen species generation [101], altered cellular ATP translocation from mitochondria inner membrane to the cytosol and increased mitochondrial uncoupling [25]. The “thrifty fuel hypothesis” suggests the anti-hyperglycemic actions of SGLT2i increases the glucagon to insulin ratio resulting in enhanced hepatic lipid mobilization and oxidation and a tonic increase in hepatic production of ketones (Figure 1.16). Ketones can serve as a fuel for myocardial ATP synthesis and do so with a more favorable ratio of ATP to oxygen [22] thus preventing negative effects associated with excessive fatty acid oxidation.

Substrate	P/O ratio***
Glucose	2.58
Pyruvate	2.50*
Palmitate	2.33**
BHOB	2.50*

Figure 1.15 ATP produced per oxygen consumed (P/O ratio) for various substrates. [111]

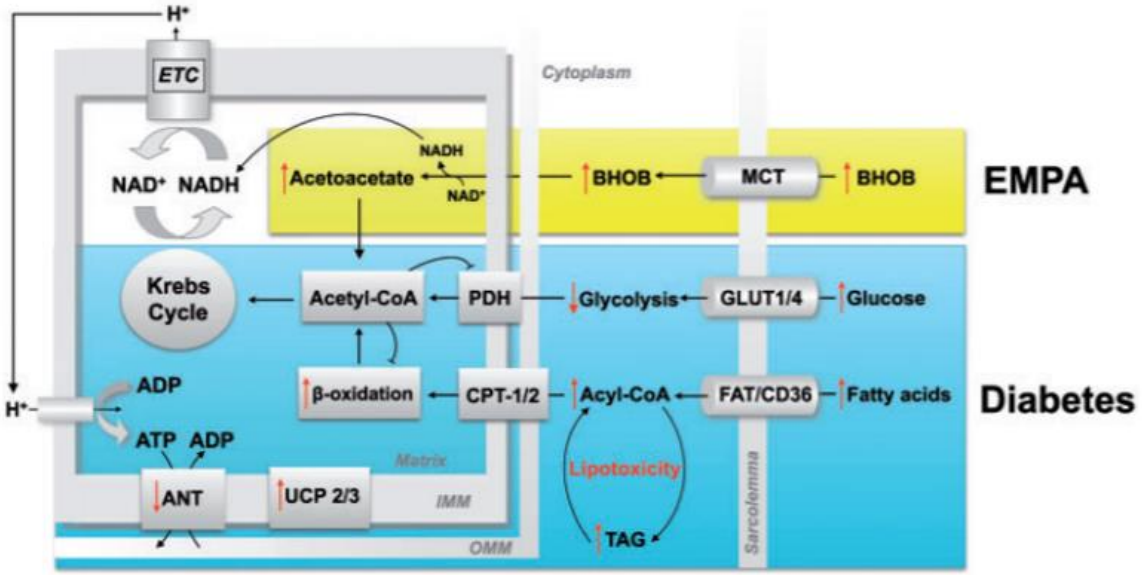


Figure 1.16 The thrifty fuel hypothesis.
Empagliflozin (EMPA) may result in an increase in a more energetically favorable substrate such as β-hydroxybutyrate (BHOB) and shift myocardial metabolism away from fatty acid utilization. ADP, adenosine diphosphate; ANT, adenine nucleotide translocase; ATP, adenosine triphosphate; CPT-1/2, carnitine palmitoyltransferase type 1/2; ETC, electron transport chain; FAT/CD36, fatty acid translocase; GLUT1/4, glucose transporter 1/4; MCT, monocarboxylate transporter; NAD^+/NADH, nicotine amide dinucleotide oxidized/reduced; PDH, pyruvate dehydrogenase; TAG, triacylglycerols; UCP 2/3, uncoupling proteins 2 and 3. [25]

Studies have been performed evaluating the effects of increased delivery of ketone bodies i.e. via a ketogenic diet or infusion of ketone bodies in pre-clinical animal and in human studies. Horton et al. evaluated the effect of a ketogenic diet 1 week prior to a TAC/MI (transverse aortic constriction combined with a small apical myocardial infarction) procedure and for four weeks post TAC/MI procedure in mice. Mice fed a ketogenic diet had increased circulating ketone levels compared to control chow fed mice. They found no differences in ejection fraction between the two groups however, they did observe significant changes in left ventricular volumes. Both left ventricular end diastolic and end systolic volumes were significantly lower in the animals on a ketogenic diet. Additionally, Horton et al. performed studies in a canine tachypacing model of dilated cardiomyopathy which has myocardial alterations consistent with dilated congestive heart failure. After 13

days of over-pacing, the ketone body 3-hydroxybutyrate (3-OHB) was infused into the right ventricle for the remainder of the protocol (day 29 of pacing). Infusion of 3-OHB prevented increases in left ventricular end diastolic pressure, a key parameter for identifying heart failure and evaluating its progression [77]. Horton et al. observed a 30% increase in myocardial mechanical energy efficiency (pressure-volume area/myocardial oxygen consumption) consistent with increased stroke work (pressure-volume area) as myocardial oxygen consumption remained unchanged compared to non-paced controls.

Nielsen et al. evaluated the effect of 3-hydroxybutyrate (3-OHB) infusion in heart failure patients with reduced ejection fraction. They demonstrated that circulating 3-OHB levels increased from 0.4 to 3.3 mM, which resulted in a significant increase in cardiac output, stroke volume, heart rate and left ventricular ejection fraction [115]. Gormsen et al. evaluated Na-3-hydroxbutyrate (ketone) infusion in 8 healthy adults and observed reduced myocardial glucose uptake, increased myocardial blood flow and no change in free fatty acid oxidation with ketone infusion compared to saline infusion [58]. Together these data provide evidence of beneficial hemodynamic effect of increased ketones in heart failure animal models and in heart failure patients. Clear evidence that SGLT2i treatment-induced shifts to ketones as a fuel source will improve or maintain cardiac function in ischemic heart disease does not yet exist.

Sodium hypothesis

Another proposed mechanism for the cardiovascular benefit is an off-target action of SGLT2i on the cardiac Na^+/H^+ exchanger, i.e. the “sodium hypothesis”. Studies in rat and rabbit cardiomyocytes have demonstrated the ability of SGLT2i to inhibit NHE-1 [19], the predominant isoform expressed in cardiac tissue [32, 125]. Under normal conditions, this exchanger is responsible for extruding protons from the myocyte and serving to regulate intracellular pH [154]. Intracellular acidosis is the major stimulus for NHE-1

activity. Both activity and expression of NHE-1 [18] have been shown to be elevated in conditions of metabolic stress such as myocardial ischemia and heart failure. This protein exchanges H^+ for Na^+ meaning that enhanced proton extrusion will result in increased Na^+ uptake [25]. Studies in isolated cardiomyocytes have demonstrated that under conditions of hyperactive NHE-1, the resultant influx of Na^+ is sufficient to affect the Na^+/Ca^{2+} exchanger (NCX). NCX transports Ca^{2+} out as Na^+ enters (i.e. forward mode of NCX). However, under the condition of increased intracellular Na^+, the NCX runs in reverse, thereby increasing intracellular Ca^{2+} [120]. SGLT2i blockade of NHE-1 would prevent the increase in cytoplasmic Na^+ and by extension prevent intracellular accumulation of Ca^{2+} and the consequent pathological hypercontractility. The net effect of the "sodium hypothesis" is depicted in Figure 1.17**.**

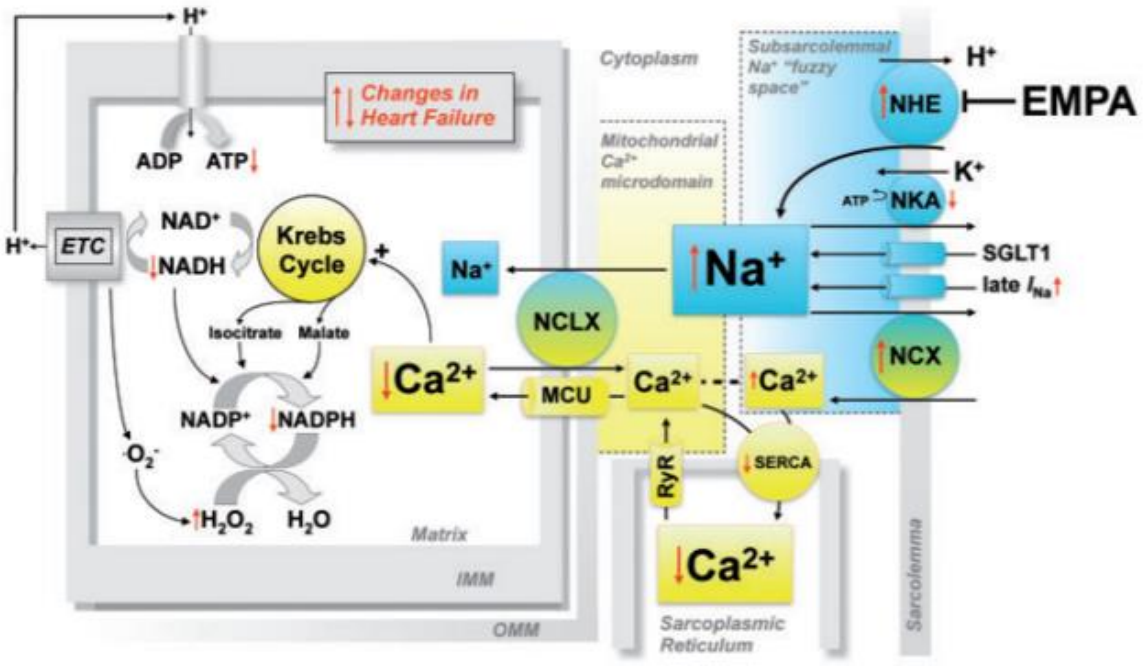


Figure 1.17 The sodium hypothesis.
EMPA may protect failing cardiac myocytes by improving Na^+ and Ca^{2+} handling via NHE inhibition. ADP, adenosine diphosphate; ATP, adenosine triphosphate; ETC, electron transport chain; MCU, mitochondrial Ca^{2+} uniporter; NAD^+ / NADH, nicotine amide dinucleotide oxidized/reduced; NCX, sarcolemmal Na^+ /Ca^{2+} exchanger; NKA, Na^+/K^+ ATPase; IMM, inner mitochondrial membrane; OMM, outer mitochondrial membrane; RyR, ryanodine receptor; SERCA, sarcoplasmic reticulum Ca^{2+} ATPase [25]

Pre-clinical studies with NHE-1i have demonstrated positive cardiovascular effects. Hartmann and Decking evaluated the NHE-1i cariporide in isolated guinea-pig hearts subjected to 15 minutes of ischemia and 30 minutes of reperfusion. They observed significant reductions in ischemia-induced rise in intracellular Na^+, improved post-ischemic contractile function and diminished contracture development with cariporide pre-treatment [67]. Fukuta et al. performed studies in open-chest swine pretreated with amiloride (NHE-1i) and subjected to 30 minutes of regional ischemia followed by 180 minutes of reperfusion. They demonstrated that pre-treatment with amiloride significantly reduced infarct size in the open-chest swine model of ischemia/reperfusion [53]. Additionally, Spitznagel et al. evaluated the effects of cariporide in a rat model of infarct-induced heart

failure. They found that chronic treatment with cariporide decreased left ventricular end diastolic pressure and improved myocardial contractility [142]. These studies provide support for NHE-1 mediated cardioprotection during ischemia/heart failure. Whether SGLT2i mediates NHE-1 activity during ischemia/heart failure as described by the "sodium hypothesis", remains to be determined.

Summary and proposed experimental aims

At present, there is a paucity of data regarding the cardiovascular effects of SGLT2i in health or disease. Recent rodent studies support SGLT2i mediated improvements in cardiac function leading to improved cardiac efficiency during myocardial ischemia. One such study by Sayour et al. demonstrated canagliflozin mediated improvements in cardiac efficiency compared to control animals in non-diabetic rats subjected to ischemia-reperfusion injury [131]. Two plausible mechanisms for the observed cardiac effects include the "thrifty fuel hypothesis" and "the sodium hypothesis" however, the exact mechanisms responsible for these effects have not been delineated. Coincidently, both myocardial metabolism ("thrifty fuel hypothesis") and Na^{+}/Ca^{2+} handling (potentially mediated by inhibition of NHE-1, "the sodium hypothesis") play an important role in determining cardiac efficiency (Figure 1.17). Furthermore, SGLT2i have been reported to increase ketone levels [39, 46, 88] and inhibit NHE-1 in isolated rodent cardiomyocytes [19, 149].

Efficiency is defined as the relationship between work performed relative to the amount of energy consumed, simply put, for work to be done energy is required [79, 136]. This holds true for the heart, for the heart to perform it's pumping function (work) it requires energy. The heart obtains most of its energy through mitochondrial oxidative phosphorylation [37]. Under normal conditions, the fuel source for the mitochondria is ~70% circulating free fatty acids and ~30% glucose [37, 101]. However, in situations such

as myocardial ischemia or heart failure, and in the setting of diabetes, there is a preferential shift in fuel selection where the heart utilizes fatty acids almost exclusively [22, 101]. Therefore, for simplicity when calculating cardiac efficiency myocardial oxygen consumption (M_VO_2) is used as a surrogate, regardless of what fuel source is being oxidized oxygen is being consumed [79, 136].

In the heart, the left ventricular external work represents the work being done by the heart and is the product of stroke volume and mean arterial pressure. Accordingly, the calculation for cardiac efficiency is stroke work / M_VO_2 or stroke volume *x* mean arterial pressure / coronary blood flow *x* arteriovenous oxygen difference. As such, myocardial substrate selection plays an important role in determining cardiac efficiency. Cardiac function is linked to oxygen supply such that cardiac function is proportionally impaired with reductions in oxygen supply [136]. However, the heart can use what oxygen is available more efficiently by altering which substrate is consumed (M_VO_2). Ketones yield more ATP per oxygen consumed compared to other substrates (have a higher P/O ratio, number of phosphates for ATP generation per molecule of oxygen consumed, Figure 1.15) and therefore are a more efficient fuel source (Figure 1.16). This generated ATP is then used for crossbridge cycling (contraction) and for ion transport (Na^+ and Ca^{2+}) as shown in Figure 1.18 [136]. Myocardial work depends on the ATP generated by oxidative phosphorylation such that cardiac efficiency is dependent on how efficient the heart is at converting ATP (by hydrolysis) to mechanical work [79]. The heart being able to maintain ATP generation or minimize the reduction of ATP generation during ischemia is critical to cardiac function and therefore cardiac efficiency.

Na^+/Ca^{2+} handling is also a major determining factor in myocardial work through regulation of excitation-contraction coupling and in modulating diastolic and systolic heart function [54]. Under normal conditions cardiac NHE-1 is quiescent and becomes active when intracellular pH is reduced as observed in ischemic conditions [18, 154]. The

increased activity of NHE-1 leads to an increased influx of Na^+ and consequent dysfunctional Ca^{2+} handling ultimately leading to diastolic and systolic dysfunction [120]. Inhibition of NHE-1 during ischemia prevents the dysregulation of Na^+ and consequent Ca^{2+} overload thereby allowing the heart to maintain cardiac function and most importantly cardiac efficiency during ischemia and reperfusion (Figure 1.17). Therefore, both the "thrifty fuel hypothesis" and the "sodium hypothesis" are plausible cardioprotective mechanisms and may not be mutually exclusive as energy levels are tied to Na^+/Ca^{2+} handling and contraction (Figure 1.18). However, neither hypothesis has been directly attributed to for SGLT2i cardioprotective effects.

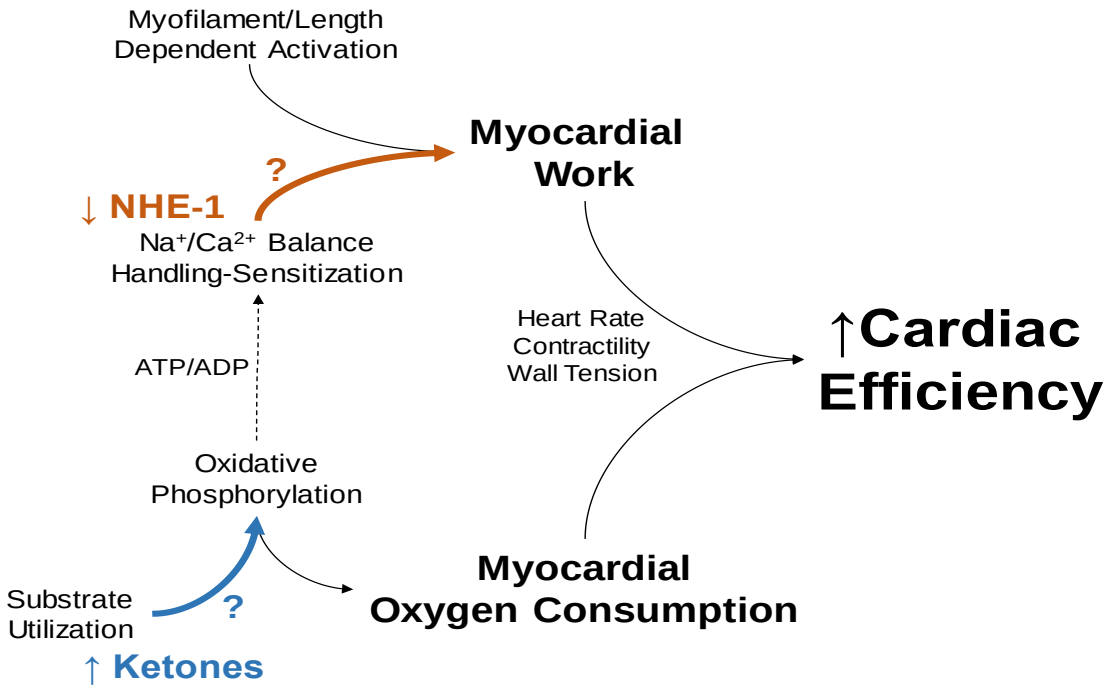


Figure 1.18 Schematic diagram of the proposed effects of SGLT2i on the primary determinants of cardiac efficiency.

The goal of this proposal is to examine the mechanisms of cardiovascular effects of SGLT2i in ischemia/reperfusion. This goal will be accomplished by addressing the following specific aims:

1. Test the hypothesis that SGLT2i improves cardiac contractile function and efficiency and attenuates infarct size in response to ischemia/reperfusion injury via salutary effects on myocardial metabolism. Integrative *in vivo* studies in open-chest swine will evaluate the relationship between left ventricular pressure and volume, myocardial substrate selection and infarct size during regional ischemia/reperfusion injury in the absence and presence of the SGLT2i. Additional *in vitro* studies will investigate the extent of SGLT2 mRNA and protein expression in swine kidney vs. heart.

2. Evaluate the hypothesis that NHE-1 contributes to the cardiac effects of SGLT2i in response to myocardial ischemia/reperfusion injury. *In vivo* studies in open-chest swine will evaluate the relationship between left ventricular pressure and volume during regional myocardial ischemia injury in the absence and presence of the NHE-1i. *In vitro* studies will examine the effects of SGLT2i on NHE1 function.

Chapter 2: Inhibition of Sodium Glucose Cotransporter-2 Preserves Cardiac Function During Regional Myocardial Ischemia Independent of Alterations in Myocardial Substrate Utilization

Introduction

The type 2 sodium-glucose co-transporter (SGLT2) is located in the S1 and S2 segments of the renal proximal tubules and is responsible for ~90% of glucose reabsorption in the kidney. In chronic hyperglycemia, SGLT2 activity is increased as a result of upregulation, exacerbating increases in plasma glucose concentration [108]. Inhibitors of this co-transporter (SGLT2i) reduce glucose reabsorption in the kidneys, resulting in increased urinary glucose excretion and a reduction in plasma glucose levels [69]. Cardiovascular outcome studies have demonstrated that SGLT2i also significantly reduce major adverse cardiovascular events in subjects with type 2 diabetes mellitus (T2DM) [12, 114, 164]. These cardiovascular benefits do not appear to be mediated by direct effects of these agents on the SGLT2 transporter in cardiovascular tissues, as SGLT2 protein expression is not detected in the heart or vasculature [32, 65, 68]. These benefits are also not explained by improved glycemic control, as anti-diabetes agents in other classes have provided similar improvements in glucose without parallel beneficial cardiac effects [104, 114, 164]. Furthermore, SGLT2i have been shown to improve cardiac function and mitigate myocardial infarct size in metabolically normal animal models [97, 129]. Improved arterial stiffness [33] and reduced blood pressure [34] have been reported with chronic SGLT2i treatment, however, it is unclear whether these effects are sufficient to explain the observed cardiovascular benefits.

In this context the ‘thrifty fuel’ hypothesis has been proposed to explain the cardiovascular benefits of SLGT2i [111]. In particular, inhibition of SGLT2 has been demonstrated to augment circulating ketone bodies via alterations in hepatic fuel

metabolism [48, 112]. Since ketone transport via the monocarboxyl transporter depends simply on the transmembrane gradient, increased ketone availability could serve to shift myocardial fuel utilization away from free fatty acids toward ketones, which require less oxygen per mole of ATP produced [22]. Some support for the plausibility of this ketone hypothesis in improving cardiac function and efficiency in response to pathologic stimuli such as ischemia has been presented [47]. However, studies to directly assess the effects of SGLT2i on myocardial substrate utilization during ischemia are presently lacking.

The goal of the present study was to evaluate the effects of SGLT2i on cardiac contractile function, substrate utilization, and efficiency before and during regional myocardial ischemia/reperfusion injury in normal, metabolically healthy swine. This study tested the hypothesis that SGLT2i improves cardiac function and efficiency during ischemia/reperfusion injury via shifts in myocardial substrate selection toward ketone utilization. To exclude contributions of effects of chronic exposure, studies were performed following a very short-term (24 hr) exposure to SGLT2i. Invasive methodologies were used to obtain high-quality and precise *in vivo* measures of hemodynamics and cardiac function, including gold standard measurements of contractility. Findings from this study provide novel insight in to the acute, cardioprotective effects of SGLT2i during ischemic injury.

Methods

Animal model and surgical preparation

All experiments involving animals were approved by an Institutional Animal Care and Use Committee and performed in accordance with the Guide for the Care and Use of Laboratory Animals (National Institutes of Health Publication. No. 85-23, Revised 2011). Male domestic swine (~50 kg) were placed on a standard chow (5L80, Purina Test Diet, Richmond, IN, USA) and randomly assigned into Control or Treatment groups. Treatment

animals were given canagliflozin (300 mg, oral) 24 hours prior to and approximately 2 hours before the acute/terminal procedure. Control animals received no treatment.

Left Circumflex Ischemia Protocol

Following an overnight fast (~16 hours), control (n = 7) and canagliflozin treated (n = 8) swine were anesthetized with telazol (5 mg/kg), ketamine (3 mg/kg), and xylazine (2.2mg/kg) cocktail (i.m.) and then anesthesia was maintained with morphine (3 mg/kg) and α-chloralose (100 mg/kg, i.v). Depth of anesthesia was monitored by observing continuous measurements of arterial blood pressure and heart rate as well as regular (15-minute intervals) reflex tests (corneal, jaw, limb withdrawal), beginning after induction of anesthesia and continuing throughout the experimental protocol. Periodic supplementation with α-chloralose was performed every 1.5 hours to maintain a level, stage 3 plane of anesthesia. The right femoral artery and vein were isolated and catheterized to allow measurement of systemic arterial pressure and maintain venous access, respectively. The heart was exposed by a left lateral thoracotomy. The proximal region of both the left anterior descending artery (LAD) and left circumflex artery (LCX) were isolated for placement of perivascular flow probes (Transonic Systems Inc., Ithaca, NY) and a snare occluder was placed around the left circumflex artery. A catheter was then placed in the intraventricular vein to allow sampling of coronary venous blood from the area supplied by the LAD. A Sci-Sense pressure/volume admittance catheter (Transonic Scisense, London Ontario, Canada) was passed through a hemostatic control valve placed directly into the left ventricle via a transmural stab near the base of the left ventricle and secured with a purse string suture. Measurements were obtained at baseline, during a 30 minute complete occlusion of the LCX, and during a 2-hour reperfusion. At the beginning of the study, a total of 20 animals were enrolled (10 per group; randomized). Three swine from the control group and two swine from the

canagliflozin group fibrillated during the coronary occlusion portion of the protocol and were thus not included in the study.

Metabolic Analysis

Arterial (5 mL) and coronary venous (5 mL) blood samples were collected simultaneously into untreated syringes, immediately sealed, and placed on ice. These samples were collected every 15 minutes during baseline and the coronary occlusion, and every 30 minutes during reperfusion; total blood samples across the 2-3-hour experimental protocol summed to <100 mL from an estimated ~4-5 L total circulating blood volume. The samples were analyzed immediately upon collection for pH, PCO_2, PO_2, O_2 content, glucose, lactate and hematocrit with an Instrumentation Laboratories automatic blood gas analyzer (GEM Premier 4000). Free fatty acids (NEFA- Non-Esterified Fatty Acids) were measured colorimetrically (WAKO Life Sciences, City State) and ketones were analyzed with a chemistry analyzer (Roche Hitachi Modular P, Indianapolis IN), reporting concentrations of β-hydroxybutyrate and acetone as total ketone measure. Myocardial oxygen consumption (MvO_2; μl O_2/min/g) was calculated using the Fick principle [coronary blood flow × (arterial O_2 content – coronary venous O_2 content)]. For these calculations, LAD perfusion territory was estimated to be 30% of total heart weight, as previously described [45]. The Fick principle was also used to calculate substrate uptake (glucose, lactate, free fatty acids, and ketone), [coronary blood flow × (arterial substrate content – coronary venous substrate content)]. Accordingly, the term “uptake” refers to the result of the Fick calculation and it should be recognized that this variable does not account for the degree to which substrate production by the tissue (e.g. lactate) contributes to the reported value. Cardiac efficiency was calculated as cardiac work (product of cardiac output and mean arterial pressure) divided by MvO_2.

Infarct protocol

Additional studies to assess the effects of SGLT2i on myocardial infarct size were also performed in control (n = 8) and canagliflozin treated (n = 8) swine. Owing to the fact that historic attempts at proximal occlusions of the LCX have resulted in high mortality rates with prolonged ischemic duration as well as variable access to distal LCX territories, these studies were performed in the distal LAD region, as previously described [55, 56]. In these experiments, the distal LAD was subjected to a 60 minute occlusion followed by 2 hours of reperfusion. Durations of ischemia and reperfusion were selected based on literature support indicating that the stimulus would be adequate and appropriate for induction of regional myocardial infarction [55, 56]. Four swine fibrillated during the LAD coronary occlusion portion of this protocol (n = 2 in each group) and thus were not included in infarct analyses. Therefore, for infarct analyses, the final n = 6 for each group.

Hemodynamic data were collected in these animals as well. However, due to a failure of the pressure volume catheter, pressure volume loops for two animals in the infarct study (n = 1 per group) were unreliable. As a result, the n for metrics associated with the pressure-volume catheter (left ventricular pressures, volumes and derived measures) is 5 per group.

Infarct Quantification

At the completion of each experiment, hearts were fibrillated by direct application of a 9V battery and excised. Hearts were then sliced into 1-cm-thick sections and incubated in 1% 2, 3,5-triphenyltetrazolium chloride (TTC) solution for 20 minutes at 37°C. Following incubation with TTC solution, a digital image of each heart slice was captured by a Canon Eos-Rebel sl1 with an associated 18-55mm lens, capturing 18.0 MP images in RAW format. No measurable infarct was detected in animals subjected to LCX occlusion regardless of treatment (control n = 3; SGLT2i n = 4) and those animals are, accordingly,

not included in infarct analyses. Measurable infarct was present and consistent in animals who received the longer (60 minute) occlusion of the distal LAD. In those animals, quantification of infarct area (unstained) versus viable myocardium (stained) was performed using ImageJ software (National Institutes of Health). Each heart slice was evaluated by three separate investigators who were blinded to experimental treatment group. Data are presented as averaged area for left ventricle (stained) and infarct area (unstained); the coefficient of variation between the three investigators was 7% for the left ventricle and 9% for the infarcted area.

RNA Isolation and cDNA synthesis

Tissue mRNA was prepared using a miRNeasy Mini Kit (Qiagen Cat#1038703). Flash frozen (500 mg) pig heart (n = 5) and kidney (n = 5) samples from domestic swine were homogenized in 2 mL Qiazol with a Polytron homogenizer (Kinematica Model PT3100) at 1000 rpm until clear homogenate was observed (1-2 min in cold room on ice bath). The homogenate was vigorously shaken for 15 seconds with 380 µL chloroform. Total RNA was isolated from the upper aqueous phase according to the kit protocol using on-column DNA digestion. cDNA was synthesized from 500 ng total RNA in a 20 µL reaction using a High Capacity cDNA Reverse Transcription Kit (Applied Biosystems Cat#4368814).

RT PCR

The pig SGLT1 RT PCR assay was obtained from ThermoFisher (Applied Bioscience catalog # Ss03374377_m1). The SGLT2 assay was designed on the basis of whole genome shotgun sequence published in NCBI (Reference Sequence: NC_010445.4) and made by Integrated DNA Technologies, Inc. (Skokie, Illinois) as a custom assay. Primer/Probe sequences were: Pig SGLT2- Assay 1, reverse primer GAA

TCC AGC TGA GCC ACT C, forward primer GGG AAG CGA TAC TGT CAG ATC, probe TGG AAA AGGACC CCT CTG GAT TTG G. The pig SGLT2-Assay 2, reverse primer GGA CAG GTA GAG GCG AAT G, forward primer CTC TTC GTG CCA GTG TAC C, probe CGC CGG TGTCAT TAC CAT GCC. cDNA was diluted 1:5 and RT-PCR was accomplished with Taqman 2X Universal PCR Master Mix (Applied Biosytems, catalog # 4304437) in ABI Prism 7900HT Sequence Detection System (Applied Biosystems). Cycles for SGLT1 and 2 were normalized to housekeeping gene, peptidylpropyl isomerase A.

Statistical Analyses

Data were analyzed using the SigmaPlot statistical package (version 11 Systat Software Inc, San Jose, CA) and SPSS (version 22 IBM, Chicago, IL, USA). Data are presented as mean ± standard error. Comparisons were assessed by t-test or two-way ANOVA; [Factor A = treatment (time control/SGLT2i); Factor B = condition (baseline/occlusion/reperfusion)] as appropriate. When significance was established with ANOVA, Student–Newman–Keuls post-hoc testing was performed to identify pairwise differences between treatment groups and conditions. Statistical significance was declared when $P < 0.05$.

Results

mRNA expression of SGLT-1 vs. SGLT-2 in swine heart and kidney

Determination of the relative abundance of SLC5A1 transcript (the gene encoding SGLT1 protein) and SLC5A2 transcript (the gene encoding SGLT2 protein) was assessed by qPCR in kidney and left ventricular biopsies from domestic swine (n = 5). These analyses detected the presence of SGLT1 (Figure 2.1A) and SGLT2 (Figure 2.1B) mRNA in both heart and kidney. However, the abundance of both of these transcripts was approximately 100-fold higher in kidney tissue than that of heart tissue.

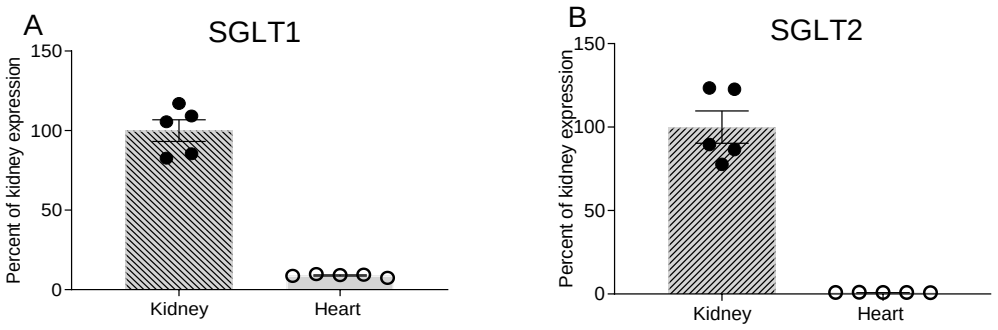


Figure 2.1 qPCR for SGLT1 (Panel A) and SGLT2 (Panel B) in kidney (n = 5) vs. heart (n = 5) biopsies from domestic swine. [20]

Systemic effects of SGLT-2i during ischemia/reperfusion injury of the LCX

Hemodynamic and blood gas data for control (n = 7) and canagliflozin (n = 8) treated swine at rest and during LCX ischemia/reperfusion injury are provided in Table 2.1. The 24-hour exposure to canagliflozin did not significantly affect baseline resting systolic arterial pressure ($P = 0.16$), diastolic arterial pressure ($P = 0.93$) or coronary blood flow ($P = 0.56$) (Table 2.1). Although canagliflozin-related decreases in arterial pH ($P < 0.001$) and hematocrit ($P < 0.001$) and increases in arterial PO_2 ($P < 0.001$) were detected by ANOVA, values of these variables remained within physiologic limits throughout the experimental protocol (Table 2.1).

Mean arterial pressure decreased from ~85 ± 5 mmHg at baseline to ~70 ± 5 mmHg following 30 minute ischemia/reperfusion injury in control and canagliflozin treated swine (Figure 2.2A; Condition: *P* = 0.03; Treatment: *P* = 0.53). Heart rate averaged ~75 beats/min throughout the entire protocol in both untreated and treated swine (Figure 2.2B**;** Condition: *P* = 0.98; Treatment group: *P* = 0.80). None of the hemodynamic or blood gas parameters were significantly affected by 30 minute ischemia/reperfusion (Table 2.1).

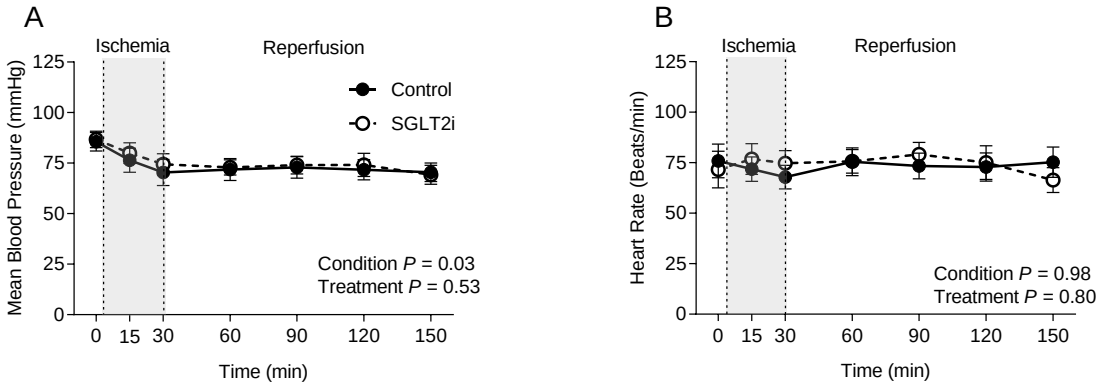


Figure 2.2 Effects of ischemia/reperfusion injury on mean blood pressure (Panel A) and heart rate (Panel B) in Control (n = 7) and SGLT2i (canagliflozin) treated (n = 8) swine. [20]

Effects of SGLT2i on cardiac contractile function during ischemia/reperfusion injury of the LCX

Under baseline conditions canagliflozin had no effect on left ventricular end diastolic volume (*P* = 0.69), end systolic volume (*P* = 0.47) stroke volume (*P* = 0.76) or cardiac output (*P* = 0.92) (Figure 2.3). However, following the onset of regional myocardial ischemia (LCX occlusion), canagliflozin treatment was associated with a significant and acute increase in left ventricular end-diastolic volume (Figure 2.3A; *P* = 0.002) and left ventricular end-systolic volume (Figure 2.3B; *P* = 0.03). These changes in cardiac volumes corresponded with a maintenance of stroke volume (Figure 2.3C; *P* = 0.03) and cardiac output (Figure 2.3D; *P* = 0.05) throughout the ischemic time period and were not observed in control animals. Canagliflozin did not affect load-dependent measures of cardiac

function, including ejection fraction (P = 0.14), dP/dt_{max} (P = 0.06), dP/dt_{min} (P = 0.30), or $Tau_{½}$ (P = 0.31) (Table 2.1). MvO_2 of the non-occluded anterior coronary vascular bed was unaffected by canagliflozin or by 30 minute ischemia/reperfusion in either group (Table 2.1).

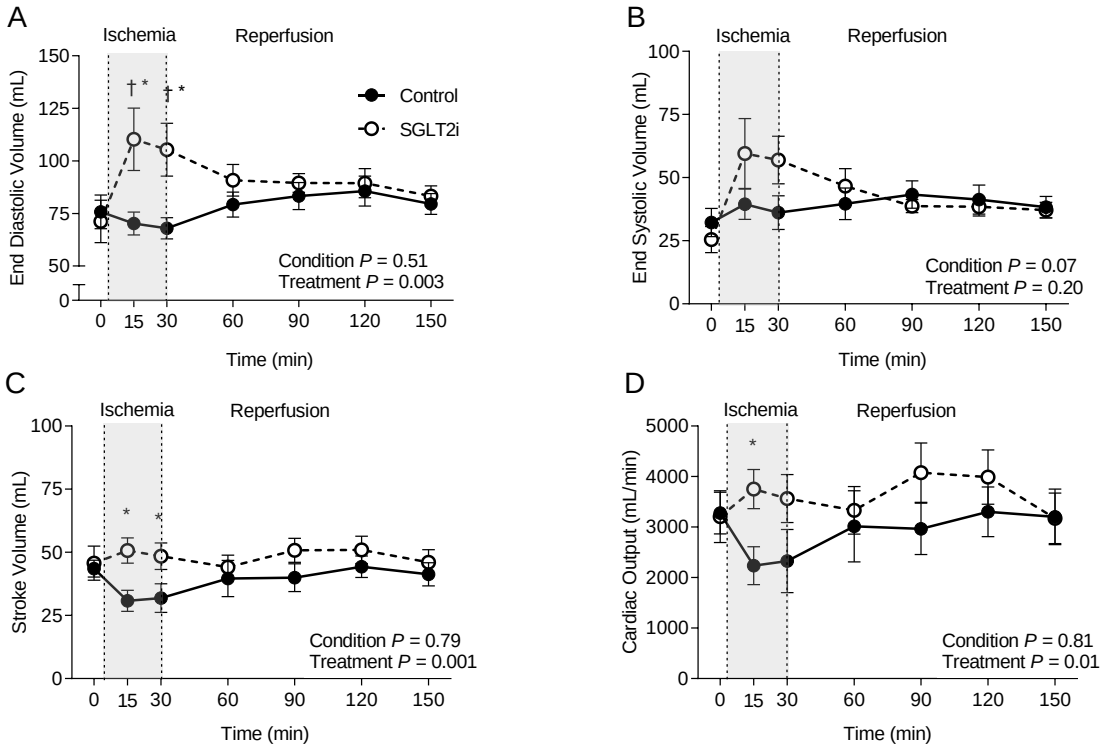


Figure 2.3 Effect of ischemia/reperfusion injury on end diastolic volume (Panel A), end systolic volume (Panel B), stroke volume (Panel C) and cardiac output (Panel D) in Control (n = 7) and SGLT2i (canagliflozin) treated (n = 8) swine. * P < 0.05 vs. Control (same time point), † P < 0.05 vs. baseline value (same treatment). [20]

Schematic representations of average steady-state left ventricular pressure-volume loops illustrating the effect of regional myocardial ischemia in control vs. canagliflozin treated swine are provided in Figure 2.4. In control swine these pressure-volume relationships demonstrate an expected modest reduction of left ventricular end-diastolic volume and systolic pressure generation during regional ischemia (Figure 2.4A). This was notably different in the canagliflozin-treated animals, which showed a substantial

right shift of the pressure-volume relationship (Figure 2.4B). Comparisons of the relationship between stroke volume vs. end-diastolic volume and cardiac output vs. end-diastolic volume (Frank-Starling relationship) during ischemia in control and canagliflozin treated swine are provided in Figure 2.4. These plots reveal that canagliflozin-mediated increases in stroke volume (Figure 2.4C) and cardiac output (Figure 2.4D) were directly related to increases in end-diastolic volume (preload). Left ventricular end diastolic pressure was unaffected by 30 minute ischemia/reperfusion or SGLT2i with canagliflozin (Table 1).

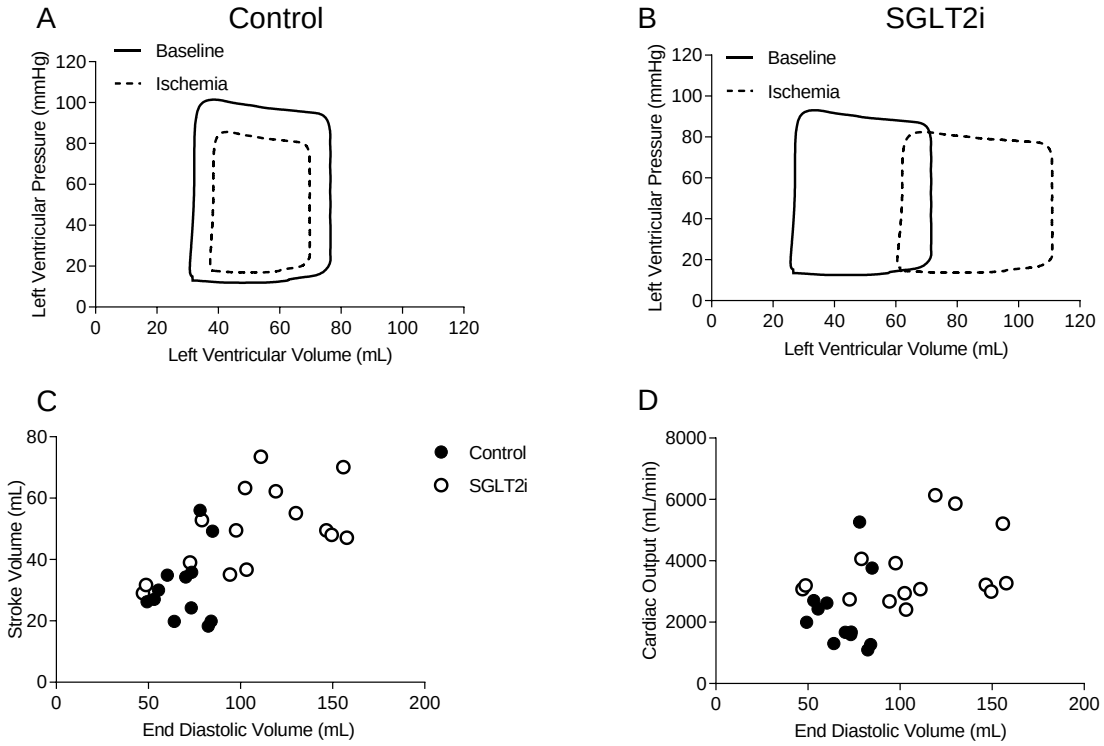


Figure 2.4 Representative pressure-volume loops of average steady state conditions at baseline and during regional myocardial ischemia in Control (n = 7) (Panel A) and SGLT2i (canagliflozin) (n = 8) treated (Panel B) swine. Relationship between stroke volume (Panel C) and cardiac output (Panel D) and end diastolic volume during ischemia in Control (n = 8) and SGLT2i (canagliflozin) (n = 7) treated swine. [20]

Stroke work (stroke volume (mL) x mean arterial pressure (mmHg)) and efficiency (cardiac output (mL/min) × mean arterial pressure (mmHg)) / MvO_2 (µl O_2/min/g) were unaffected by canagliflozin treatment under baseline conditions (Figure 2.5). However, stroke work (Figure 2.5A; $P < 0.001$) and efficiency (Figure 2.5B; $P = 0.03$) were significantly increased by canagliflozin administration during regional myocardial ischemia compared to control swine.

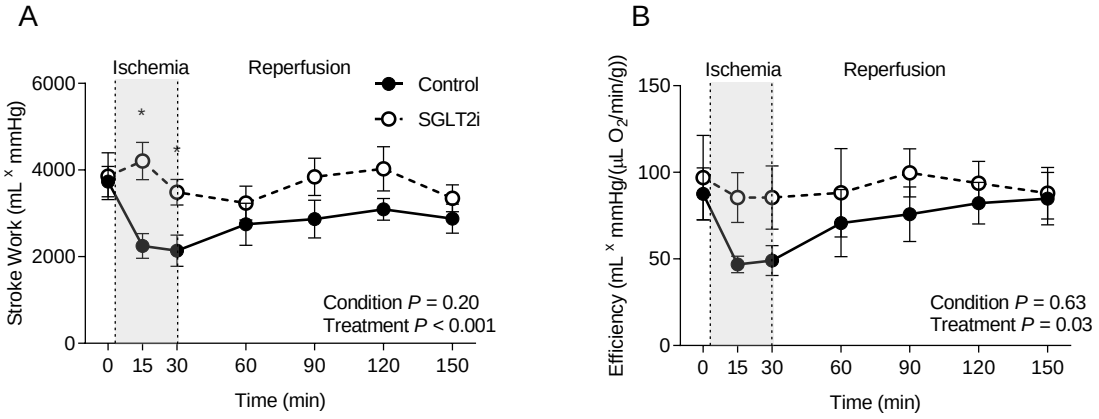


Figure 2.5 Effects of ischemia/reperfusion injury on cardiac stroke work (Panel A) and efficiency (Panel B) in Control (n = 7) and SGLT2i (canagliflozin) treated (n = 8) swine. * $P < 0.05$ vs. Control (same time point). [20]

Effects of SGLT2i on myocardial substrate selection during ischemia/reperfusion injury of the LCX

Administration of canagliflozin augmented arterial free fatty acid concentration (Table 2.2**;** $P = 0.017$). However, circulating concentrations of other cardiac substrates including glucose ($P = 0.087$), lactate ($P = 0.336$) and total ketone bodies (acetoacetone and 3-hydroxybutyrate) ($P = 0.74$) were unaffected by SGLT2i (Table 2.2). Canagliflozin did not significantly influence myocardial uptake of glucose ($P = 0.75$), lactate ($P = 0.26$), ketones ($P = 0.41$) or free fatty acids ($P = 0.31$) (Figure 2.6), under resting conditions, during the 30 min ischemia, or during reperfusion.

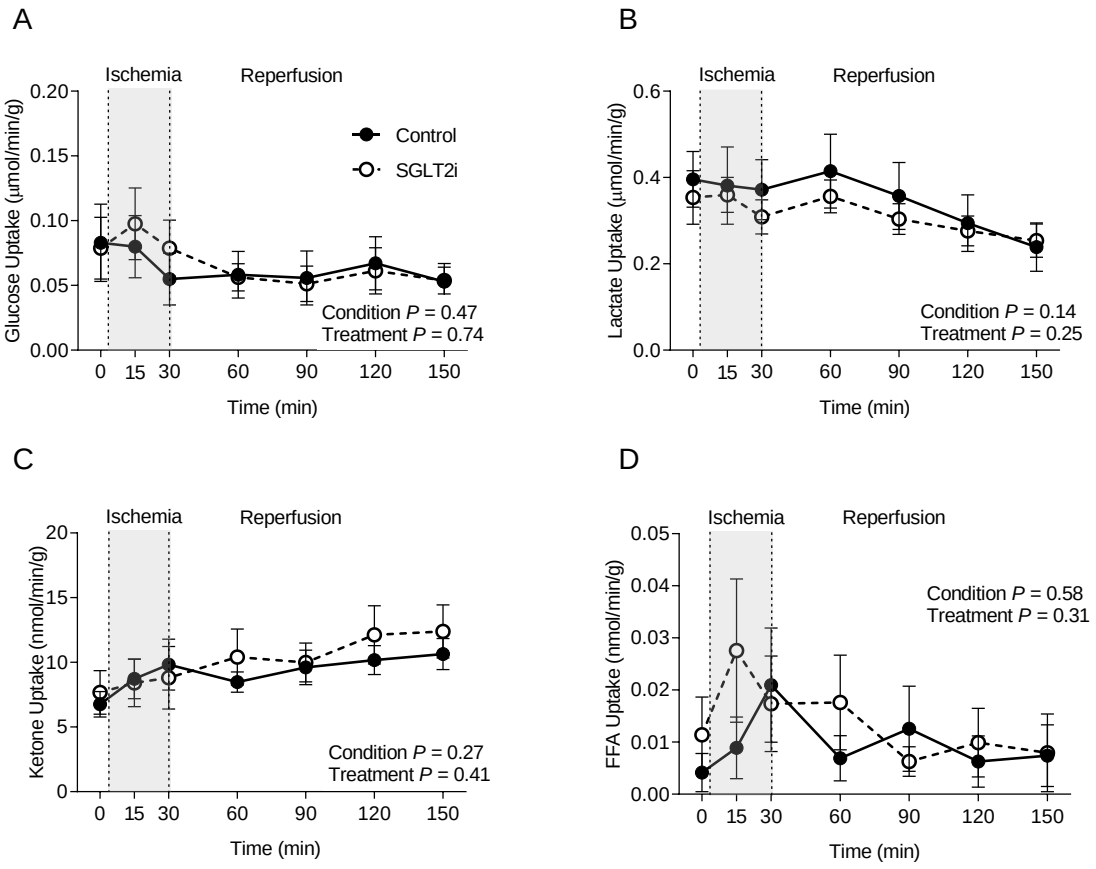


Figure 2.6 Effect of ischemia/reperfusion injury on myocardial uptake of glucose (Panel A), lactate (Panel B), ketones (Panel C) and free fatty acids (FFA) (Panel D) in Control (n = 7) and SGLT2i (canagliflozin) treated (n = 8) swine. [20]

Effect of SGLT2i on myocardial infarct size and cardiac function in response to 60-minute LAD occlusion

No evidence of myocardial infarction was detected in either control (n = 3) or SGLT2i (n = 4) treated hearts following the 30 minute LCX occlusion protocol. Therefore, to better assess potential effects on magnitude of myocardial infarction, a 60 minute occlusion with 2-hour reperfusion was performed in a separate cohort of animals in the distal LAD region. The rationale for a change in perfusion territory was based on historic attempts at longer term proximal occlusions of the LCX which resulted in marked increases in mortality [55, 56]. Therefore, our additional cohort of animals, was modeled

after established protocols demonstrating reliable induction of myocardial infarction with higher survival rates following 60 minute occlusion of the distal LAD in swine [55, 56].

In contrast to data from the 30 minute LCX occlusion, TTC staining of hearts from the 60 minute LAD occlusion protocol revealed that treatment with canagliflozin (n = 6) significantly reduced infarct size by ~60% compared to control (n = 6) swine (Figure 2.7; $P = 0.03$). Additionally, canagliflozin treatment produced similar effects following the onset (15 and 30 minute) of the distal LAD occlusion, including a significant increase in left ventricular end-diastolic volume and stroke volume (Appendix B; $P = 0.01$) compared to control swine. Heart rate was significantly lower in the SGLT2i group at the later ischemic time points (≥ 30 minute occlusion) (Appendix A). However, this reduction was not associated with alterations in end diastolic volume or stroke volume at either 45 or 60min occlusion.

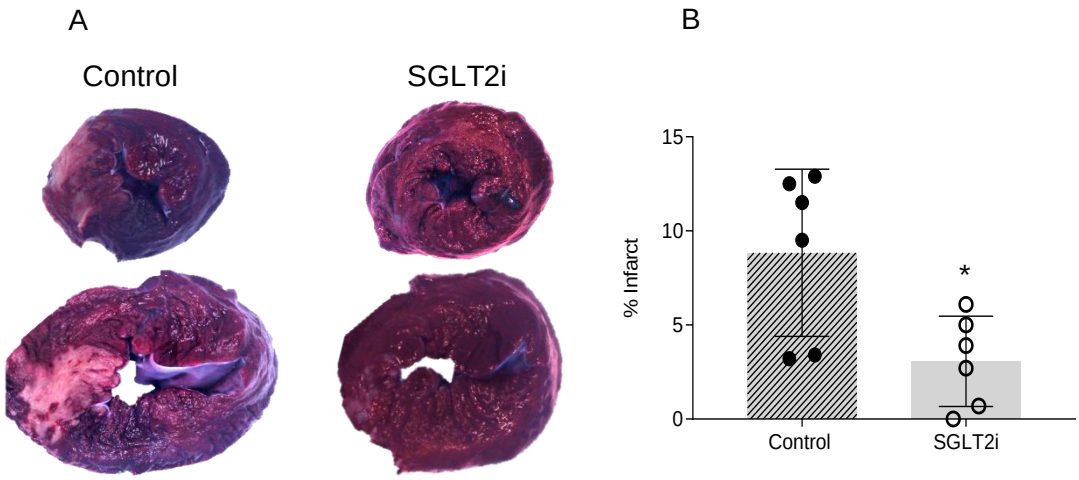


Figure 2.7 Images in Panel A show representative transmural sections of left ventricular slices from control (n = 6) and canagliflozin (n = 6) treated swine. Quantification of total infarct area relative to total left ventricular area is presented in Panel B. * $P < 0.05$ vs. control. [20]

Discussion

There is a growing body of evidence supporting beneficial cardiovascular effects of SGLT2i in the setting of obesity/type 2 diabetes [12, 114, 164] and in normal, metabolically healthy animal models [97, 129]. However, there was no *a priori* reason to expect such beneficial effects of these glucosuric agents, and the mechanisms underpinning these unexpected effects are unknown. As such, whether effects of SGLT2i are the result of direct action on cardiomyocytes or other cardiac cells or, are secondary to extra-cardiac effects of SGLT2i remains undetermined. A proposed mechanism to explain SGLT2i-associated improvements in major adverse cardiovascular events as well as reductions in myocardial infarct size [12, 97] centers around the "thrifty fuel hypothesis" [22, 49, 112, 143]. This hypothesis is based on a recognized effect of SGLT2i to induce ketone production, which by providing ketones to the heart may improve cardiac efficiency by shifting myocardial substrate utilization away from free fatty acids [22, 47]. Accordingly, this study was designed to evaluate the effects of the SGLT2i canagliflozin on cardiac contractile function, substrate utilization, and efficiency before and during regional myocardial ischemia/reperfusion injury in normal, metabolically healthy swine. Effects of canagliflozin to augment cardiac function during regional myocardial ischemia and reduce myocardial infarct size were observed in this study, but these effects were independent of alterations myocardial substrate utilization. These observations argue against contributions of ketone-induced shifts in fuel selection to the cardioprotective effects of SGLT2i.

Cardiac effects of SGLT2i

Here, shown for the first time that SGLT2i produces a prominent and sustained increase in left ventricular volumes during regional myocardial ischemia in otherwise normal, metabolically healthy swine. There was no evidence of these effects under

baseline conditions, and they abated during reperfusion (Figure 2.4). Further, these effects were not associated with any changes in arterial blood pressure (afterload) or heart rate (ventricular filling time) (Figure 2.2). These data are consistent with recent rodent investigations which demonstrate that SGLT2i was able to preserve cardiac function in pressure-overload induced heart failure mice independent of changes in afterload, as cardioprotection was evident both *in vivo* and in subsequent isolated heart studies [29].

Significant increases in end diastolic volume could be interpreted as potentially deleterious, as volume overload is commonly associated with dilated cardiomyopathy and/or exacerbation of myocardial infarction. In this study, however, changes in ventricular volume handling were seen only during ischemia and reversed with relief of ischemia, at least under these acute exposure conditions. The timeline of the ischemia in this study (minutes) would not allow for the necessary remodeling associated with a dilated cardiomyopathy phenotype. Further, interpretation of this study is simplified as effects were evident in the absence of measurable myocardial infarction, as measured by TTC staining in the 30 minute LCX occlusion study. The lack of myocardial infarction following a 30 minute coronary occlusion is consistent with previously published studies in swine [55, 71, 72, 132]. Therefore, changes in ventricular volume handling must occur independent of ventricular remodeling and/or infarction. The observations of unchanged end diastolic pressure suggest that SGLT2i enhanced ventricular compliance; however, no significant differences in global indices of diastolic function (dP/dt_{min}, Tau) were noted.

Although any evidence of myocardial infarction following the 30 minute LCX occlusion protocol were not observed, insights into potential infarct mitigating behavior of SGLT2i are fundamentally valuable. Therefore, additional studies were performed in a separate cohort of animals wherein conditionally identical swine were subjected to a 60 minute coronary artery occlusion. The LAD was occluded during these experiments as longer duration LCX occlusion is associated with decreased survivability [55]. In these

animals, a significant SGLT2i associated reduction in infarct size was demonstrated (Figure 2.7). This finding is consistent with prior studies in rodent models which documented reductions in myocardial infarct size in response to ischemia/reperfusion injury [12, 97].

Small amounts of SGLT1 and SGLT2 mRNA were detected from swine myocardial samples (Figure 2.1), consistent with observations in human samples [32, 65, 68]. However, it is unclear whether the SGLT2i mediated effects in this study are the result of direct and/or indirect actions on the heart. As initial dosing was 24 hours prior to the acute study, so the potential for paracrine/humoral mediation exists. The 300 mg oral dosing regimen utilized in this study was selected to directly parallel the "high-dose" utilized clinically. Prior studies indicate that a single oral dose of canagliflozin (300 mg) achieves a plasma C_{max} of 3,480 ng/mL (7.67 μM) in humans with a t_{max} of 1.5 hours and a half-life of 14.9 hours [41, 42, 133]. We estimate (using allometric scaling) that the maximal plasma concentration of canagliflozin in ~50 kg swine such as those used in the present study to be ~3757 ng/mL (assuming no plasma protein binding), which is comparable to exposure in humans [41, 42, 133]. Given the present observations of clear effects of canagliflozin on myocardial function it seems plausible that a myocardial receptor system might exist that is responsive to this agent but not from the SGLT2 protein family; one potential target, the sodium hydrogen exchanger-1 (NHE-1) is discussed below. Therefore, further studies to determine dose-dependence and direct vs. indirect actions of SGLT2i are needed.

Potential mechanisms underlying cardiac effects of SGLT2i

SGLT2i therapies have been demonstrated to augment circulating ketone bodies in type 2 diabetes thereby suggesting the "thrifty fuel hypothesis" of cardioprotection. In this study canagliflozin increased free fatty acid concentrations modestly but did not affect arterial levels of lactate or ketone bodies (Table 2.2). Arterial glucose levels tended to be

lower in the treatment group (P = 0.08) however, ischemia/reperfusion status had no effect on glucose levels on either group (P = 1.0). These data are consistent with prior studies of SGLT2i treatment in metabolically healthy humans who also exhibited no change in fasting plasma glucose or ketone concentrations [7]. Despite the modest changes in circulating substrate concentration that we observed, no changes in myocardial uptake of glucose, lactate, ketones, or free fatty acid were seen (Figure 2.6). Thus, while interventions such as insulin and malonyl CoA inhibition have been shown to improve contractile function during ischemia via promotion of the utilization of thrifty fuels [49, 90, 144], this does not appear to be the explanation for the currently observed effects of SGLT2i. A caveat of the present study is that findings were made in a metabolically normal animal model, under conditions of acute and controlled ischemia. Therefore, whether these findings extend to the setting of chronic treatment in patients with type 2 diabetes with or without acute ischemia merits future study.

It is well recognized that SGLT2i influence circulating blood volume, promoting osmotic diuresis and natriuresis and reductions in plasma volume [33, 86]. Although did plasma volume was not directly measured, there was a modest but significant decrease in hematocrit in SGLT2i treated swine which runs counter to anticipated effects of diuresis. Further, the current observations are not consistent with beneficial effects of diuresis, as any reduction in plasma volume would be expected to reduce overall ventricular filling volumes, in contrast to our observed ~50% increase in end diastolic volume during ischemia. Also, no differences in hemodynamics or cardiac volumes were noted at baseline or during the reperfusion period (Figure 2.3). Cardiovascular benefits associated with diuretic-induced reductions in plasma volume are understood to result from preload lowering and associated decreased wall tension within the ventricle and direct reductions in Frank-Starling mediated cardiac force generation [36], again in contrast to what was

observed. Together, these data argue against a role of diuresis in the observed effects of canagliflozin following short term (single-day) therapy and acute ischemic insult.

An alternate hypothesis to potentially explain SLGT2i mediated cardioprotection is the “sodium hypothesis”. Recent evidence suggests that the SGLT2i mediated effects could work via direct inhibition of NHE-1 as has been demonstrated in isolated cardiomyocytes from rats and rabbits [19, 149]. In conditions leading to cellular acidosis such as myocardial ischemia, NHE-1 has been shown to be activated resulting in a reversal of the Na^+/Ca^{2+} exchanger and increase intracellular calcium [116, 120]. By extension, inhibition of NHE-1 would act to lessen Ca^{2+} overload in cardiomyocytes during myocardial ischemia. Improvement in cytosolic Ca^{2+} handling also produces myriad effects on regulatory and contractile proteins within the cardiomyocyte [9, 59, 102, 128, 155]. As the interplay between developed tension and resting cardiomyocyte length can determine Starling effects [110], a mechanism that directly alters intracellular Ca^{2+} handling could potentially contribute to the effects seen in this investigation. Previous studies have demonstrated a reduction in infarct size in a swine model of ischemia/reperfusion with NHE-1 inhibitors [89]. These data are directionally consistent with our present observations and suggest a direction for further systematic *in vivo* and *in vitro* studies to explore the potential mechanisms of SGLT2i-mediated cardioprotection.

Conclusion

Data from these studies demonstrate that canagliflozin preserves cardiac contractile function and efficiency during acute regional myocardial ischemia through acute effects on cardiac volume regulation that cannot be explained by myocardial fuel switching. Further, SGLT2i reduces infarct size following 60 minutes of ischemia. Findings from this study highlight the therapeutic potential for SGLT2i in metabolically normal subjects [129] and demonstrate the need for further investigations to both elucidate the

mechanisms responsible for improvements in cardiac function during ischemia and to extend these studies into metabolically deranged cohorts as well as cohorts with normal glucose metabolism but abnormal cardiac function.

Table 2.1

Effects of canagliflozin on systemic hemodynamics, cardiac contractile function and blood gas parameters

Parameter	Treatment	Condition							Condition *P*-value	Treatment *P*-value	Interaction *P*-value
		Baseline	Ischemia		Reperfusion						
		0 min	15 min	30 min	60 min	90 min	120 min	150 min			
Arterial pH	Control	7.57 ± 0.01	7.56 ± 0.01	7.55 ±0 .01	7.54 ± 0.01	7.53 ± 0.01	7.55 ± 0.01	7.55 ± 0.10	0.753	<0.001	0.895
	SGLT2i	7.52 ± 0.01	7.52 ± 0.01	7.52 ± 0.01	7.49 ± 0.02	7.52 ± 0.02	7.52 ± 0.01	7.50 ± 0.03 †			
Arterial PCO_2 (mmHg)	Control	38 ± 2	38 ± 2	39 ± 2	40 ± 2	41 ± 2	39 ± 2	39 ± 2	0.993	0.953	0.907
	SGLT2i	40 ± 1	39 ± 1	39 ± 1	39 ± 1	38 ± 1	38 ± 1	41 ± 1			
Arterial PO_2 (mmHg)	Control	143 ± 12	143 ± 11	143 ± 11	136 ± 9	138 ± 10	139 ± 10	134 ± 9	0.978	<0.001	0.873
	SGLT2i	159 ± 11	164 ± 9	167 ± 10	163 ± 7	168 ± 7	155 ± 13	179 ± 15 †			
Coronary Venous PO_2 (mmHg)	Control	16 ± 1	14 ± 2	15 ± 1	15 ± 2	15 ± 2	15 ± 2	15 ± 1	0.982	0.132	1.000
	SGLT2i	16 ± 1	15 ± 1	16 ± 1	17 ± 1	17 ± 1	16 ± 1	17 ± 2			
HCT (%)	Control	34 ± 1	35 ± 1	35 ± 1	35 ± 1	35 ± 1	34 ± 1	34 ± 1	0.978	<0.001	0.988
	SGLT2i	32 ± 1	32 ± 1	32 ± 1	33 ± 1	32 ± 1	33 ± 1	33 ± 1			
Arterial O_2 Content (mL/dL)	Control	15 ± 1	15 ± 1	15 ± 1	16 ± 1	15 ± 1	15 ± 1	15 ± 1	0.637	0.774	0.710
	SGLT2i	15 ± 1	15 ± 1	15 ± 1	16 ± 1	15 ± 1	15 ± 1	16 ± 1			
Coronary Venous O_2 Content (mL/dL)	Control	3.4 ± 0.4	2.9 ± 0.4	3. 0 ± 0.4	3.1 ± 0.5	3.1 ± 0.5	3.2 ± 0.5	3.2 ± 0.5	0.907	0.292	0.975
	SGLT2i	3.3 ± 0.3	2.9 ± 0.3	3.0 ± 0.2	3.8 ± 0.9	3.9 ± 0.9	3.6 ± 0.7	3.6 ± 0.8			
Myocardial Oxygen Consumption (µL O_2/min/g)	Control	47 ± 5	49 ± 5	43 ± 5	43 ± 6	44 ± 6	41 ± 5	39 ± 5	0.673	0.214	1.000
	SGLT2i	50 ± 7	53 ± 6	48 ± 6	47 ± 6	45 ± 6	45 ± 6	43 ± 4			
Systolic Pressure (mmHg)	Control	105 ± 7	94 ± 8	87 ± 8	88 ± 7	91 ± 8	90 ± 7	89 ± 7	0.362	0.02	0.989
	SGLT2i	127 ± 17	111 ± 14	106 ± 14	103 ± 14	101 ± 11	99 ± 11	95 ± 10			
Diastolic Pressure (mmHg)	Control	71 ± 4	64 ± 5	58 ± 5	60 ± 4	61 ± 4	60 ± 4	59 ± 4	0.095	0.468	0.995
	SGLT2i	71 ± 3	67 ± 5	63 ± 5	62 ± 3	63 ± 5	63 ± 5	57 ± 4			
Systemic Vascular Resistance ($mmHg^x$min/mL)	Control	0.02 ± 0.00	0.03 ± 0.00	0.04 ± 0.00	0.03 ± 0.00	0.03 ± 0.00	0.02 ± 0.00	0.02 ± 0.00	0.832	0.03	0.557
	SGLT2i	0.03 ± 0.00	0.02 ± 0.00	0.02 ± 0.00	0.02 ± 0.00	0.02 ± 0.00	0.02 ± 0.00	0.02 ± 0.00			
Coronary Blood Flow (ml/min/g)	Control	0.40 ± 0.05	0.38 ± 0.05	0.34 ± 0.05	0.35 ± 0.05	0.35 ± 0.05	0.34 ± 0.05	0.33 ± 0.05	0.673	0.063	1.000
	SGLT2i	0.44 ± 0.06	0.44 ± 0.05	0.40 ± 0.05	0.40 ± 0.04	0.39 ± 0.04	0.39 ± 0.03	0.36 ± 0.03			

Ejection Fraction (%)	Control	59 ± 3	45 ± 5	47 ± 7	49 ± 7	48 ± 5	52 ± 4	52 ± 5	0.069	0.146	0.984
	SGLT2i	64 ± 4	50 ± 6	48 ± 4	50 ± 5	56 ± 3	57 ± 4	55 ± 4			
Left Ventricular End Diastolic Pressure (mmHg)	Control	14 ± 2	16 ± 2	16 ± 2	15 ± 2	15 ± 2	14 ± 2	13 ± 2	0.886	0.624	0.999
	SGLT2i	13 ± 2	16 ± 2	15 ± 2	14 ± 2	13 ± 2	14 ± 2	14 ± 3			
dP/dt_{max} (mmHg/s)	Control	1748 ± 138	1358 ± 191	2296 ± 895	1368 ± 158	1330 ± 143	1405 ± 131	1322 ± 112	0.120	0.066	0.487
	SGLT2i	1522 ± 191	1307 ± 140	1235 ± 125	1212 ± 134	1264 ± 125	1262 ± 131	1336 ± 99			
dP/dt_{min} (mmHg/s)	Control	-1613 ± 168	-1251 ± 168	-1162 ± 131	-1246 ± 229	-1164 ± 171	-1161 ± 192	-1101 ± 163	0.031	0.641	0.446
	SGLT2i	-1708 ± 183	-1471 ± 109	-1199 ± 109	-1127 ± 102	-1111 ± 109	-1155 ± 133	-999 ± 102			
$Tau_{1/2}$ (ms)	Control	30 ± 3	38 ± 5	41 ± 5	37 ± 5	40 ± 8	43 ± 9	45 ±10	0.234	0.414	0.857
	SGLT2i	31 ± 3	30 ± 1	34 ± 3	39 ± 4	39 ± 5	35 ± 5	37 ± 3			
$Tau_{1/e}$ (ms)	Control	21 ± 2	25 ± 3	28 ± 4	25 ± 3	28 ± 6	30 ± 7	32 ± 8	0.214	0.382	0.870
	SGLT2i	21 ± 2	20 ± 1	23 ± 2	27 ± 3	27 ± 3	24 ± 3	26 ± 2			

Values are mean ± SE for Control (n = 7) and SGLT2i (canagliflozin) (n = 8). † $P < 0.05$ vs. baseline value (same treatment) [23]

Table 2.2

Effects of canagliflozin on circulating substrate concentrations

Parameter	Treatment	Condition							Condition *P*-value	Treatment *P*-value	Interaction *P*-value
		Baseline	Ischemia		Reperfusion						
		0 min	15 min	30 min	60 min	90 min	120 min	150 min			
Arterial Glucose (mg/dL)	Control	104 ± 14	110 ± 21	105 ± 19	120±24	119 ± 26	112 ± 21	109 ± 20	1.000	0.087	0.990
	SGLT2i	102 ± 25	96 ± 20	93 ± 20	91±20	84 ± 18	89 ± 20	89 ± 19			
Arterial Lactate (mmol/L)	Control	2.0 ± 0.4	2.5 ± 0.7	2.8 ± 0.9	3.1 ± 0.9	3.0 ± 0.8	2.7 ± 0.7	2.4 ± 0.6	0.754	0.336	1.000
	SGLT2i	1.8 ± 0.4	2.1 ± 0.5	2.2 ± 0.5	2.8 ± 0.8	2.5 ± 0.7	2.5 ± 0.6	2.2 ± 0.5			
Arterial Ketone (µmol/L)	Control	31 ± 5	41 ± 8	48 ± 10	47 ± 8	50 ± 9	53 ± 8	59 ± 10	0.002	0.74	0.953
	SGLT2i	31 ± 4	34 ± 5	37 ± 6	46 ± 6	49 ± 6	59 ± 7	63 ± 8 *			
Arterial FFA (mmol/L)	Control	0.32 ± 0.06	0.32 ± 0.05	0.31 ± 0.05	0.34 ± 0.06	0.34 ± 0.05	0.35 ± 0.06	0.36 ± 0.05	0.995	0.017	0.999
	SGLT2i	0.43 ± 0.05	0.41 ± 0.05	0.44 ± 0.05	0.44 ± 0.06	0.47 ± 0.06	0.49 ± 0.07	0.50 ± 0.08			

Values are mean ± SE for Control (n = 7) and SGLT2i (canagliflozin) (n = 8). * $P < 0.05$ vs. control (same time point) [23]

Chapter 3: Inhibition of Sodium Glucose Cotransporter-2 Improves Cardiac Efficiency During Regional Myocardial Ischemia Independent of Sodium/Hydrogen Exchanger-1

Introduction

Sodium glucose co-transporter 2 inhibitors (SGLT2i) are a class of anti-hyperglycemic agents that reduce circulating blood glucose levels by increasing urinary glucose excretion [69]. Recent clinical outcome studies demonstrated unexpected and significant decreases in major adverse cardiac events (MACE) with SGLT2i therapy, independent of effects on HbA1c [114, 158, 164]. Notably, SGLT2i therapy is associated with decreases in heart failure related hospitalization, non-fatal myocardial infarction and cardiovascular death [114, 158, 164]. Moreover, this effect is not limited to a single compound in the category as is demonstrable by the results of the study of 10,142 participants in the Canagliflozin Cardiovascular Assessment Study (CANVAS) [114] and 7,020 participants in the multicenter Empagliflozin, Cardiovascular Outcomes, and Mortality in Type 2 Diabetes trial (EMPA-REG) [164]. However, the specific effects and mechanisms by which SGLT2i potentially influence the cardiovascular system remain poorly defined.

Multiple mechanisms to explain the cardiovascular benefits of SGLT2i have been proposed. At present the two most plausible are the “thrifty fuel hypothesis” and the “sodium hypothesis” which propose an SGLT2i mediated shift in myocardial substrate utilization toward ketone bodies vs. the direct inhibition of the sodium hydrogen exchanger-1 (NHE-1) respectively. Recent data from our investigative team demonstrated that SGLT2i augments ventricular filling, maintains myocardial efficiency, and attenuates infarct size in response to acute ischemia-reperfusion injury independent of alterations in myocardial substrate selection, volume loading, or afterload in lean healthy swine [20].

These findings are consistent with investigations from other groups [29, 96, 131] and argue against a requisite role for ketone utilization as the sole mechanism for SGLT2i-mediated improvements in cardiovascular function in the acute ischemia setting. Alternatively, recent evidence in isolated cardiomyocytes has demonstrated that NHE-1 activity is directly inhibited by SGLT2i [19, 149]. While prior studies support NHE-1i improves post-ischemic contractile dysfunction [67] and attenuates myocardial infarct size [53], there are at present a paucity of data to address the extent to which SGLT2i-mediated cardioprotection occurs via effects on NHE-1.

The goal of the present study was to test the hypothesis that NHE-1 contributes to the cardiac effects of SGLT2i in response to myocardial ischemia-reperfusion injury. Experiments evaluated whether acute administration of the SGLT2i canagliflozin (30 μM) vs. the NHE-1i cariporide (1 μM) influence cardiac contractile function and efficiency in a directionally consistent manner. Studies were performed in lean, metabolically healthy open-chest swine with high fidelity pressure-volume admittance catheters. Complementary fluorometric NHE-1 activity assays were also were performed in wild-type CHO (AP-1) cells transfected with human NHE-1. Findings from this study provide novel mechanistic insight into the acute, cardioprotective effects of SGLT2i and NHE-1i in the setting of myocardial ischemia-reperfusion injury.

Methods

Animal model and surgical preparation

All experiments involving animals were approved by an Institutional Animal Care and Use Committee and performed in accordance with the Guide for the Care and Use of Laboratory Animals (National Institutes of Health Publication. No. 85-23, Revised 2011). Male domestic swine (~50 kg) were placed on a standard chow (5L80, Purina Test Diet, Richmond, IN, USA) and randomly assigned into control or treatment groups. Control

swine received a 15-30-minute infusion of vehicle (DMSO; n = 6), the treatment animals received a 15-30-minute infusion of either SGLT2i Canagliflozin (30 µM; n = 6), or the NHE-1 inhibitor Cariporide (1 µM; n = 6) immediately prior to total occlusion of the left circumflex artery.

Left Circumflex Ischemia Protocol

Following an overnight fast (~16 hours), swine were anesthetized with telazol (5 mg/kg), ketamine (3 mg/kg), and xylazine (2.2 mg/kg) cocktail (i.m.) and then anesthesia was maintained with morphine (3 mg/kg) and α-chloralose (100 mg/kg, i.v). Depth of anesthesia was monitored by observing continuous measurements of arterial blood pressure and heart rate as well as regular (15-minute intervals) reflex tests (corneal, jaw, limb withdrawal), beginning after induction of anesthesia and continuing throughout the experimental protocol. Periodic supplementation with α-chloralose was performed every 1.5 hours to maintain a level, stage 3 plane of anesthesia. The right femoral artery and vein were isolated and catheterized to allow measurement of systemic arterial pressure and maintain venous access, respectively. The heart was exposed by a left lateral thoracotomy. The proximal region of both the left anterior descending artery (LAD) and left circumflex artery (LCX) were isolated for placement of perivascular flow probes (Transonic Systems Inc., Ithaca, NY) and a snare occluder was placed around the left circumflex artery. A catheter was then placed in the intraventricular vein to allow sampling of coronary venous blood from the area supplied by the LAD. A Sci-Sense pressure/volume admittance catheter (Transonic Scisense, London Ontario, Canada) was passed through a hemostatic control valve placed directly into the left ventricle via a transmural stab near the base of the left ventricle and secured with a purse string suture. Measurements were obtained at baseline, during a 60 min complete occlusion of the LCX, and during a 2-hour reperfusion. At the beginning of the study, a total of 23 animals were

enrolled. Three swine died during instrumentation independent of treatment and two animals from the control group fibrillated during the coronary occlusion portion of the protocol and were thus not included in the study. Therefore, a total of n = 18 animals were included in the study (n = 6 per group, randomized).

Metabolic Analysis

Arterial (5 mL) and coronary venous (5 mL) blood samples were collected simultaneously into untreated syringes, immediately sealed, and placed on ice. These samples were collected every 15 minutes during baseline and the coronary occlusion, and every 30 minute during reperfusion; total blood samples across the 2-3-hour experimental protocol summed to <100 mL from an estimated ~4-5 L total circulating blood volume. The samples were analyzed immediately upon collection for pH, PCO_2, PO_2, O_2 content, glucose, lactate and hematocrit with an Instrumentation Laboratories automatic blood gas analyzer (GEM Premier 4000). Free fatty acids (NEFA- Non-Esterified Fatty Acids) were measured in a colorimetric assay (WAKO Life Sciences, City State) and ketones were analyzed with a chemistry analyzer (Roche Hitachi Modular P, Indianapolis IN), reporting concentrations of β-hydroxybutyrate and acetone as total ketone measured. Myocardial oxygen consumption (MvO_2; µl O_2/min/g) was calculated using the Fick principle [coronary blood flow × (arterial O_2 content – coronary venous O_2 content)]. For these calculations, LAD perfusion territory was estimated to be 30% of total heart weight, as previously described [45]. Cardiac efficiency was calculated as cardiac stroke work [stroke volume (mL) x mean arterial pressure (mmHg)] divided by MvO_2.

NHE-1 Activity Assay in Wild type NHE-1 transfected AP-1 cells

Wild type NHE-1 transfected AP-1 cells (a mutant cell line derived from Chinese hamster ovarian cells that that lack an endogenous Na+/H+ exchanger) were cultured and grown at 37^{o}C on glass cover slips until confluent. Double pulse fluorometric assays using PTI Delta Scan 4000 fluorometers were then used to test for NHE activity [11]. The assays first involved a 20-minute incubation of the cover slips at 37^{o}C in serum free growth medium with the addition of 3 µM of BCECF-AM which is used as an indicator of cytosolic pH. Intracellular pH was then measured with a PTI (Photon Technologies International) Delta-scan spectrofluorometer placed in a thermostatically controlled chamber. Coverslips containing cells were placed in 2.5 ml Na^{+} normal buffer (135 mM NaCl, 5 mM KCl, 1.8 mM $CaCl_2$, 1.0 mM $MgSO_4$, 5.5 mM Glucose, 10 mM Hepes, pH: 7.3) for 3 minutes to stabilize the pH*i*. Dual excitation at 440 nm and 502.5 nm as well as emission at 528.7 nm during the assays (2,3) were used to give pH measurements that were independent of dye concentration or cell number. To test the effect of inhibitors, a dual pulse assay was used in which two acid loads were created and recovered from consecutively, in the same set of cells (3). The rate of recovery of the second pulse plus or minus SGLT2i or NHE1i, was compared to the first pH measurement. Each NHE recovery activity was measured after a 3-minute acid load using 50 mM NH_4Cl and a 30 second exposure to sodium free buffer (135 mM N-Methyl-D-glucamine, 5.0mM KCl, 1.8 mM $CaCl_2$, 1.0 mM $MgSO_4$, 5.5 mM glucose, 10 mM Hepes, solution pH: 7.3) for 10 – 20 seconds at 37°C. Cells were allowed to recover from the acid load by incubating them in Na^{+} normal buffer for 3 – 4 minutes. Each drug was added directly into the Na^{+} containing recovery buffer (second recovery) and the rates of pH change per second with and without the drug were compared for each trial. The drugs were added upon the second pulse and so the rate of recovery 2 (ROR2) contained drug while the first pulse (ROR1) contained no drug. Six replicates were performed for each concentration of each drug tested. The following concentrations were

tested: 1, 3, 10, 30, 100, and 300 µM for CANA. and 0.01, 0.03, 0.1, 0.3, 1, 3, and 10 µM for cariporide. A negative control was performed using the same volume of DMSO solvent added to the Na^+ containing buffer with the drugs as it was used to dissolve the drugs for testing. NHE-1 activity was calculated by taking the change in cytosolic pH/s (slope) from the drug exposed recovery and dividing it by the Na^+ buffer only recovery for each trial. This was performed using the ROR slopes derived from the raw graphical data of the assays. These calculations are represented as rate of recovery 2/ rate of recovery 1 (ROR2/ROR1). The resulting NHE activities were then standardized with the negative control (0 µM of drug, DMSO only) acting as 100% activity for comparison across all concentrations. For every pH assay, a calibration curve was done at 3 successive pH's using nigericin as described earlier [10].

NHE-1 Activity Assay in iCell™ human IPSc-derived cardiomyocytes

Cryopreserved iCell™ human IPSc-derived cardiomyocytes (Cellular Dynamics International, Inc) were thawed, plated, and maintained following the manufacturer's protocol. Briefly, 20,000 cardiomyocytes/well were attached to 0.1% Gelatin (StemCell Technologies) coated 96-well plates for 48 hrs in iCell™ Cardiomyocytes Plating Medium and maintained for 14 days in iCell™ Cardiomyocytes Maintenance Medium. To facilitate intracellular pH (pHi) measurement, the iCell™ cardiomyocytes were incubated with 5 µM fluorescent pH indicator dye BCECF AM (Invitrogen) (Live Cell Imaging Solution, pH 7.4, Thermofisher Scientific) for 15 minutes, followed by a subsequent wash with HEPES buffered Ringer Solution (pH7.4) containing 140 mM NaCl, 5 mM KCl, 1mM $MgCl_2$, 1 mM $CaCl_2$, 5 mM glucose, 10 mM N-2-hydroxyethylpiperazin-N′-2-ethanesulfonic acid (HEPES). IPSc-derived cardiomyocytes were then treated with vehicle (DMSO 0.1% in HBRS, pH 7.4), cariporide or canagliflozin. The following concentrations of both cariporide and canagliflozin were tested: 0.12, 0.37, 1.1, 3.3, and 10 µM. HEPES buffered Ringer

Solution (pH 7.4), cells were exposed to HEPES buffered Ringer Solution (pH 7.4) for 5 minutes after which the buffer was then replaced by HEPES buffered Ringer Solution (pH 6.4) for 5 minutes to obtain intracellular acidification. Subsequent intracellular re-alkalinization was induced by replacing HBRS (pH 6.4) with HBRS (pH 7.4). Respective treatments were present during each step. Successive images of four fields per well under 10x objective (excitation at 405nm and 488 nm, emission at 500-550 nm) were captured immediately after each treatment was added and serial images were acquired every 1.5 minutes thereafter. Images were captured with a temperature-controlled (37°C) Opera Phenix™ High Content Screening System (PerkinElmer). Intracellular pH calibrations were performed at the end of each experiment with an Intracellular pH Calibration Buffer Kit (Thermofisher Scientific), fluorescence ratio of 488nm/405nm was calibrated to reflect pHi.

Statistical Analyses

Data were analyzed using the SigmaPlot statistical package (version 11 Systat Software Inc, San Jose, CA) and SPSS (version 22 IBM, Chicago, IL, USA). Data are presented as mean ± standard error. Comparisons were assessed by t-test or two-way ANOVA with repeated measures; [Factor A = treatment (time control/SGLT2i); Factor B = condition (baseline/occlusion/reperfusion)] as appropriate. When significance was established with ANOVA, Student–Newman–Keuls post-hoc testing was performed to identify pairwise differences between treatment groups and conditions. Statistical significance was declared when $P < 0.05$. Multiple linear regression and analysis of covariance were used to compare the slopes and intercepts of the relationships between stroke volume vs. end-diastolic volume, and stroke work vs. myocardial oxygen consumption.

Results

Systemic effects of SGLT-2i and NHE-1i during ischemia/reperfusion injury

Blood gas and hemodynamic data for control (n = 6), canagliflozin (n = 6) and cariporide (n = 6) treated swine at rest and during LCX ischemia/reperfusion injury are provided in Table 3.1 and Table 3.2 respectively. The 15-30-minute acute infusion of canagliflozin or cariporide did not significantly affect baseline resting systolic arterial pressure, diastolic arterial pressure or coronary blood flow (Table 3.2). Mean arterial pressure decreased from ~91 ± 3 mmHg at baseline to ~62 ± 3 mmHg following 60 min ischemia/reperfusion injury in control, canagliflozin and cariporide treated swine (Table 3.2; Condition: $P < 0.001$; Treatment: $P = 0.36$). Heart rate averaged ~78 beats/min at baseline and throughout the ischemic period, however, SGLT2i significantly increased heart rate during reperfusion compared to control and NHE-1i treated swine (Table 3.2**;** Condition: $P < 0.001$; Treatment: $P = 0.006$). None of the blood gas (Table 3.1) or hemodynamic parameters (Table 3.2) were significantly affected by 60 minutes ischemia or 2 hours of reperfusion. In addition, canagliflozin and cariporide had no effect on circulating concentrations of cardiac substrates including glucose ($P = 0.66$), lactate ($P = 0.46$), free fatty acids ($P = 0.12$) and total ketone bodies (acetoacetone and 3-hydroxybutyrate) ($P = 0.77$) compared to control at any time during the study (Table 3.3).

Effects of SGLT2i and NHE-1i on cardiac contractile function during ischemia/reperfusion injury

Under baseline conditions canagliflozin and cariporide had no effect on left ventricular end diastolic volume, end systolic volume, stroke volume, or cardiac output (Figure 3.1). However, following the onset of regional myocardial ischemia (LCX occlusion), canagliflozin treatment was associated with a significant increase in left ventricular end diastolic volume (Figure 3.1A; $P = 0.02$). This SGLT2i-mediated increase

in cardiac filling volume corresponded with a significant increase in stroke volume relative to control animals (Figure 3.1C; $P = 0.03$), however, no significant differences in cardiac output were detected (Figure 3.1D; $P = 0.33$). Heart rate averaged ~78 beats/min at baseline and throughout the ischemic period, however, SGLT2i significantly increased heart rate during reperfusion compared to control and NHE-1i treated swine (Table 3.2). Left ventricular end diastolic volume and stroke volume were not significantly different throughout the experimental protocol in either the control or cariporide treated animals. Neither canagliflozin nor cariporide influenced load-dependent measures of cardiac function, including ejection fraction ($P = 0.77$), dP/dt_{max} ($P = 0.92$), dP/dt_{min} ($P = 0.97$), or $Tau_{½}$ ($P = 0.18$) (Table 3.2). MvO_2 of the non-occluded LAD coronary vascular bed was unaffected by canagliflozin or cariporide treatment. (Table 3.1).

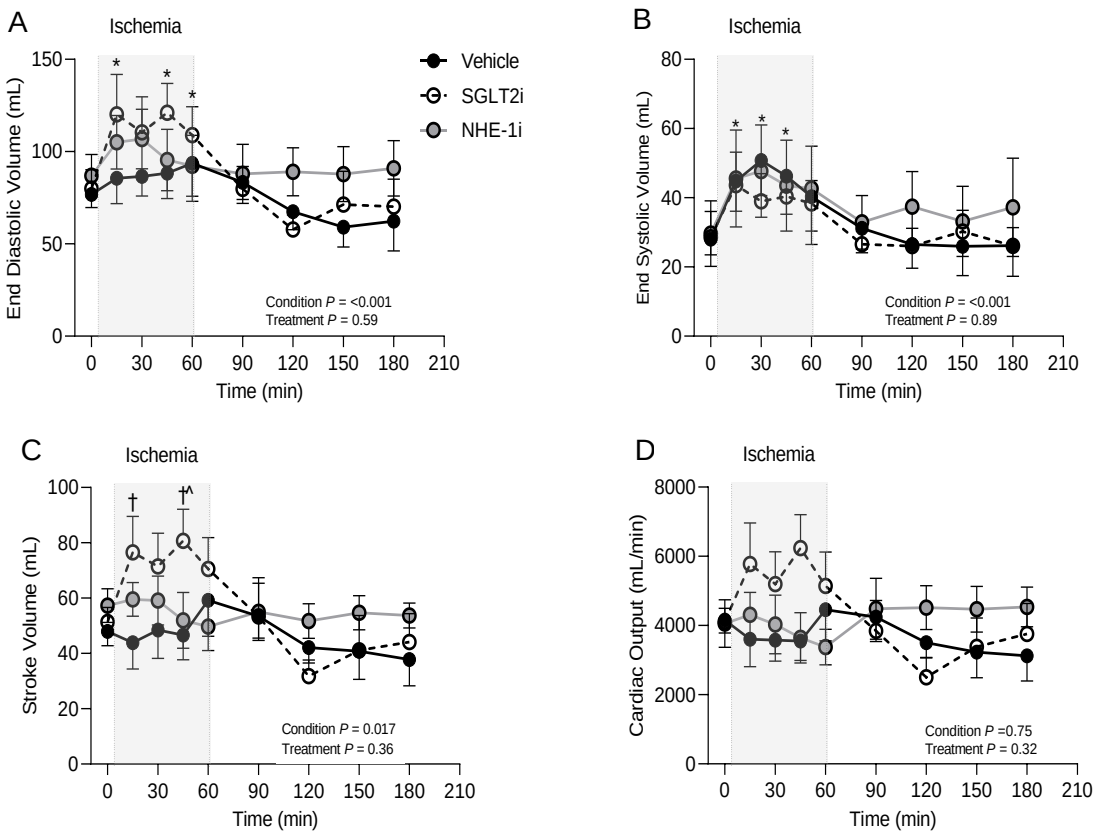


Figure 3.1 Effect of ischemia/reperfusion injury on end diastolic volume (Panel A), end systolic volume (Panel B), stroke volume (Panel C) and cardiac output (Panel D) in Control (n = 6), SGLT2i (canagliflozin) treated (n = 6) and NHE-1i(cariporide) treated (n = 6) swine. * $P < 0.05$ vs. Control (same time point), † $P < 0.05$ vs. baseline value (same treatment), ^ $P < 0.05$ vs. NHE-1i (same time point).

Schematic representations of average steady-state left ventricular pressure-volume loops illustrating the effect of regional myocardial ischemia in control, canagliflozin and cariporide treated swine are provided in Figure 3.2. Comparisons of the Frank-Starling relationship between stroke volume vs. end diastolic volume during ischemia reveal canagliflozin-mediated increases in stroke volume were directly related to increases in end-diastolic volume (preload) (Figure 3.2D; $P = 0.004$). This effect was not present in cariporide treated animals as demonstrated in Figure 3.2E. Left ventricular end diastolic pressure were unaffected by 60 minutes of ischemia/reperfusion or by SGLT2i canagliflozin or NHE-1i cariporide (Table 3.1).

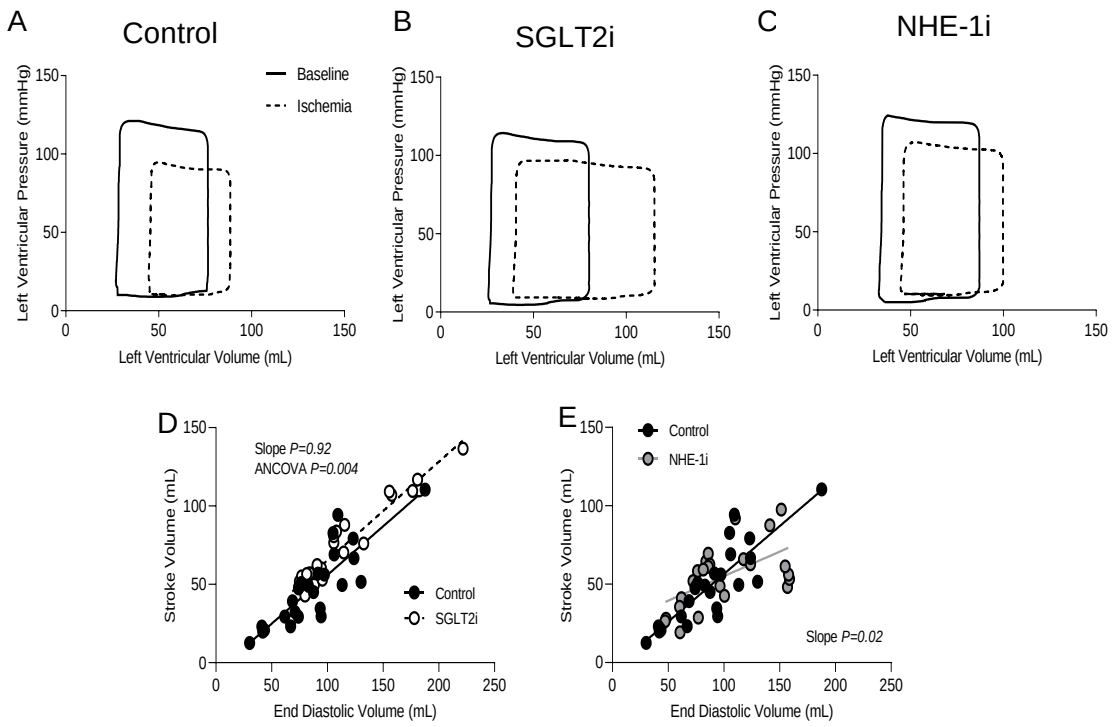


Figure 3.2 Representative pressure-volume loops of average steady state conditions at baseline and during regional myocardial ischemia in Control (n = 6) (Panel A), SGLT2i (canagliflozin) (n = 6) treated (Panel B) and NHE-1i (cariporide) (n = 6) treated (Panel C) swine. Relationship between stroke volume and end diastolic volume during ischemia in Control (n = 6) (R^2 = 0.7) and SGLT2i (canagliflozin) (n = 6) (R^2 = 0.9) (Panel D) and Control (n = 6) and NHE-1i (cariporide) (n = 6) (R^2 = 0.3) (Panel E) treated swine.

Stroke work [stroke volume (mL) x mean arterial pressure (mmHg)] and efficiency (stroke work / MvO_2 (µl O_2/min/g)) were unaffected by canagliflozin or cariporide treatment under baseline conditions (Figure 3.3). However, canagliflozin significantly increased stroke work (Figure 3.3A; *P* = 0.025) and cardiac efficiency (Figure 3.3B; *P* = 0.008) during regional myocardial ischemia compared to control swine. Cariporide did not alter stroke work (*P* = 0.37) or cardiac efficiency (*P* = 0.15) at any time point. Comparisons of the relationship between stroke work and MvO_2 during ischemia reveal a canagliflozin-mediated increase in stroke work occurred independent of any changes in MvO_2 (Figure 3.3C). Cariporide had no effect on this relationship as demonstrated in Figure 3.3D.

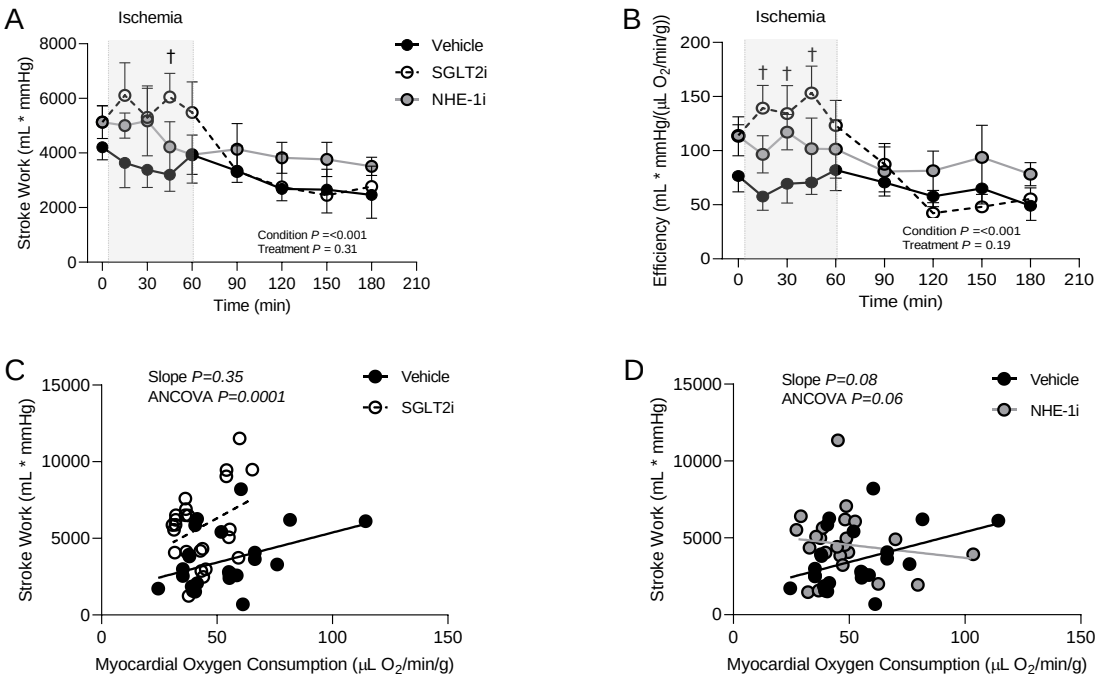


Figure 3.3 Effects of ischemia/reperfusion injury on cardiac stroke work (Panel A) and efficiency (Panel B) in Control (n = 6), SGLT2i (canagliflozin) treated (n = 6) and NHE-1i (cariporide) treated (n = 6) swine. Relationship between stroke work and myocardial oxygen consumption during ischemia in Control (n = 6) (R^2 = 0.16) and SGLT2i (canagliflozin) (n = 6) (R^2 = 0.13) (Panel D) and Control (n = 6) and NHE-1i (cariporide) (n = 6) (R^2 = 0.02) (Panel E) treated swine. † $P < 0.05$ vs. Control (same time point).

Effect of SGLT2i on NHE-1 activity in wild type AP-1cells transfected with NHE-1

Fluorometric activity assays in wild type AP-1 cells transfected with NHE-1 demonstrated concentration-dependent inhibition of NHE-1 activity by cariporide beginning at 0.03 µM continuing to a plateau to near zero % activity at 3µM (Figure 3.4A). The IC_{50} value of cariporide was 0.11µM. In contrast, canagliflozin did not cause significant changes of NHE-1 activity, with mean values of % activity ranging from 90.8% to 109.9% (Figure 3.4B). IC_{50} values for canagliflozin were not calculated due to the lack of adequate inhibitory effects.

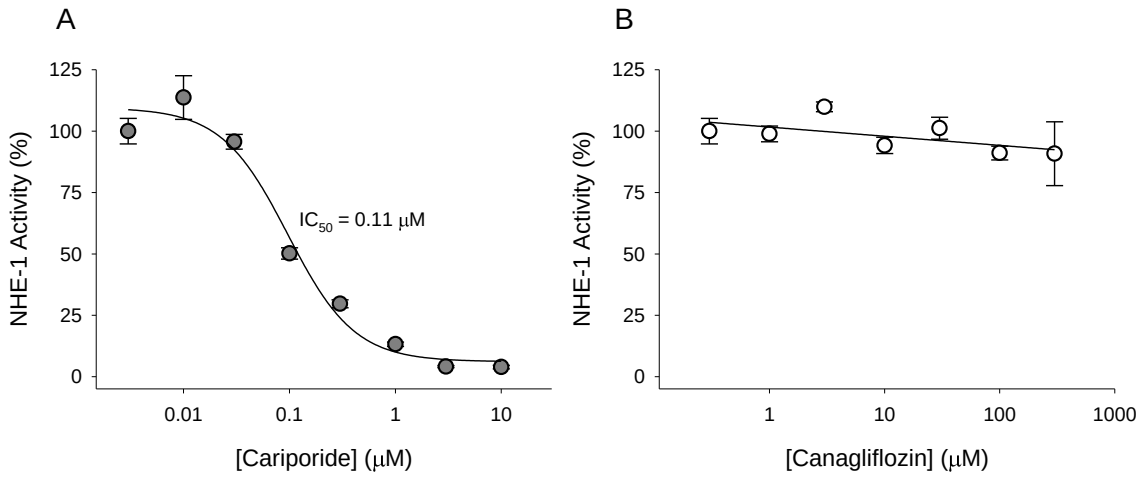


Figure 3.4 Effect of cariporide (Panel A) and canagliflozin (Panel B) on NHE-1 activity in wild type NHE-1 transfected AP-1 cells.

Effect of SGLT2i on NHE-1 Activity Assay in iCell™ human IPSc-derived cardiomyocytes

The fluorometric activity assays demonstrated concentration-dependent inhibition of NHE-1 activity by cariporide beginning at 1.1 μM and reaching near zero % activity at 10μM (Figure 3.5A). In contrast, canagliflozin had no inhibitory effect on NHE-1 (Figure 3.5B).

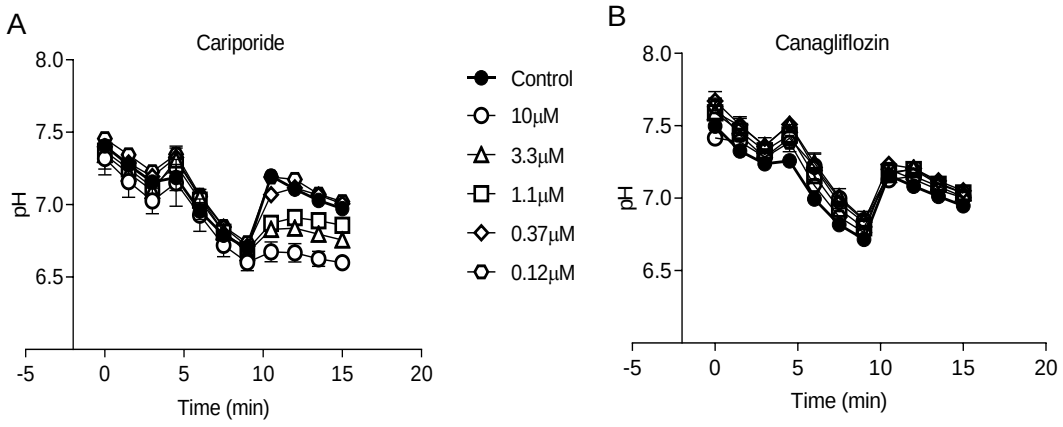


Figure 3.5 Effect of cariporide (Panel A) and canagliflozin (Panel B) on NHE1 activity in iCell™ human IPSc-derived cardiomyocytes.

Discussion

This study was designed to test the hypothesis that NHE-1 contributes to the cardiac effects of acute SGLT2i in response to myocardial ischemia-reperfusion injury. Consistent with recent findings from our laboratory [20], 15-30 minutes pre-treatment with canagliflozin increased left ventricular end diastolic volume, stroke work and improved cardiac work efficiency relative to untreated control hearts during the ischemic period. In contrast, cariporide did not significantly affect ventricular diastolic filling, stroke volume or work efficiency at any time point. Additionally, the observed cardiac effects of canagliflozin were independent of any change in circulating substrate concentration compared to control at any time during the study protocol. The distinct physiologic effects of SGLT2i vs. NHE-1i *in vivo* were corroborated by *in vitro* activity assays in which canagliflozin failed to affect NHE-1 activity of AP-1 cells transfected with human NHE-1 or in human IPSc derived cardiomyocytes. Taken together, these findings fail to support the hypothesis that the acute cardiac effects of SGLT2i are mediated via an NHE-1 dependent pathway.

Cardiac effects of SGLT2i and NHE-1i

The rationale for the so-called “sodium hypothesis” is based primarily on recent evidence in isolated cardiomyocytes which demonstrated that NHE-1 activity is directly inhibited by SGLT2i [19, 149]. Inhibition of NHE-1 under conditions of cellular acidification, such as ischemia, would prevent the increase in cytoplasmic Na^+ and by extension prevent intracellular accumulation of Ca^{2+} and the consequent pathological hypercontractility. While prior studies support NHE-1i improves post-ischemic contractile dysfunction [67] and attenuates myocardial infarct size [53], the extent to which SGLT2i-mediated cardioprotection occurs via effects on NHE-1 remains poorly defined. Here for the first time a head to head comparison of the cardiac effects of SGLT2i (canagliflozin) and NHE-1i (cariporide) in response to myocardial ischemia/reperfusion injury in normal,

metabolically healthy swine is provided. Consistent with prior data from our laboratory, significant and sustained increases in left ventricular end diastolic volume during myocardial ischemia with an acute administration (15-30 minutes pre-treatment) of canagliflozin were demonstrated (Figure 3.1A). This increased cardiac filling in SGLT2i treated animals was directly related to increased stroke work and improved efficiency as shown in Figure 3.3. These observed findings were only evident during the ischemic period and returned to baseline levels upon reperfusion. Additionally, these effects were in absence of any alteration of arterial blood pressure (afterload) or heart rate (ventricular filling time) during ischemia. The expectation would be if SGLT2i was involved with cardiac NHE-1 inhibition then treatment with an NHE-1i would demonstrate similar cardiac effects during myocardial ischemia/reperfusion. However, 15-30 minutes pre-treatment with cariporide did not alter ventricular filling volume, cardiac output or work efficiency at any time point during the study period. One could argue that the lack of observed effects with cariporide could be to be related to the chosen dose. In the present study a plasma concentration of ~1 µM was chosen based on published data demonstrating that this concentration is sufficient for effective inhibition of NHE activity in cardiac ventricular myocytes [15, 89]. Furthermore, prior studies have established that 1 µM cariporide reduced ischemia induced arrhythmias in rats and mitigates myocardial infarct size in both rabbits and pigs [16, 89, 100].

SGLT2i effects on NHE-1 activity

To further investigate the hypothesis that the cardiac effects of SGLT2i occur via inhibition of NHE-1, cellular studies evaluating the effect of canagliflozin on NHE-1 activity in response to acid loading conditions (which resemble conditions in cardiomyocytes during ischemia) were conducted. To circumvent the confounding effects of endogenous exchangers, wild type NHE-1 gene was transfected in antiport-deficient (AP-1) cells [141].

This approach allowed for evaluation of a direct effect of canagliflozin on NHE-1 activity. The NHE-1 activity studies demonstrated a dose-dependent inhibition of NHE-1 activity by cariporide as expected. Conversely, canagliflozin at all concentrations tested did not significantly attenuate NHE-1 activity (Figure 3.4). Similar studies also failed to show an effect of SGLT2i on NHE-1 activity human IPSc derived cardiomyocytes (Figure 3.5). It has previously been demonstrated that SGLT2i (empagliflozin, canagliflozin and dapagliflozin) reduced pH recovery following an acid load (measure of NHE-1 activity) in rabbit and mouse cardiomyocytes by binding NHE-1 [19, 149]. This finding contrasts with the present pig experimental data where canagliflozin and cariporide demonstrate different cardiac effects during ischemia and the NHE-1 activity assay data demonstrating no effect of canagliflozin on NHE-1 activity. Reasons for these discrepant findings remain unclear but are likely related to underlying differences between species, cell types utilized, and/or experimental conditions. Nevertheless, the cardiac findings in the present study are consistent with data from the NHE-1 activity assays in both the wild type NHE-1 transfected AP-1 cells and the IPSc derived cardiomyocytes. Furthermore, despite assay format differences, both the wild type NHE-1 transfected AP-1 cells and the IPSc derived cardiomyocytes demonstrate a concentration-dependent inhibition of NHE-1 activity with cariporide while canagliflozin had no effect on NHE-1 activity at any concentration tested.

Conclusion and Implications

The findings from this study fail to support the hypothesis that the acute cardiac effects of SGLT2i are mediated via an NHE-1 dependent pathway. Multiple mechanisms to explain the benefits have been proposed, including anti-hypertensive effects [103, 105], sodium and fluid loss [13, 138], enhanced arterial compliance [50, 94, 150], and anti-inflammatory capacity [28, 70, 161]. However, based on the acute nature of SGLT2i administration in this study (15-30 minutes) these mechanisms are unlikely to explain the

Frank-Starling effect of SGLT2i observed during ischemia. Furthermore, data from our recent study as well as the present investigation argue against a requisite role for either ketones or NHE-1 in mediating cardiac effects of SGLT2i in normal metabolically healthy swine. These findings contrast prior evidence for ketone utilization in obese heart failure suggesting mechanisms of SGLT2i may differ depending the on duration of treatment, underlying pathophysiologic conditions and metabolic status, all of which should be further explored. Regardless, the acute SGLT2i-mediated preservation of cardiac function in the absence of changes in substrate utilization or evidence of NHE-1 activity strongly suggest the involvement of other pathways that are independent of SGLT2 as neither its mRNA nor protein have been detected in cardiac tissue [32, 43].

Table 3.1

Effects of canagliflozin and cariporide on blood gas parameters

Parameter	Treatment	Condition									Condition *P*-value	Treatment *P*-value	Interaction *P*-value
		Baseline	Ischemia				Reperfusion						
		0 min	15 min	30 min	45 min	60 min	30 min	60 min	90 min	120 min			
Arterial pH	Control	7.49 ± 0.00	7.44 ± 0.03	7.45 ± 0.03	7.44 ± 0.03	7.44 ± 0.02	7.46 ± 0.02	7.46 ± 0.02	7.47 ± 0.02	7.47 ± 0.03	0.08	0.65	0.97
	SGLT2i	7.46 ± 0.03	7.45 ± 0.03	7.45 ± 0.03	7.45 ± 0.01	7.46 ± 0.01	7.47 ± 0.01	7.46 ± 0.01	7.45 ± 0.02	7.46 ± 0.03			
	NHE-1i	7.45 ± 0.03	7.46 ± 0.03	7.47 ± 0.03	7.47 ± 0.02	7.47 ± 0.01	7.48 ± 0.02	7.50 ± 0.02	7.49 ± 0.03	7.49 ± 0.03			
Arterial PCO2 (mmHg)	Control	46 ± 2	49 ± 5	45 ± 3	48 ± 4	45 ± 2	44 ± 2	44 ± 3	43 ± 1	44 ± 2	0.20	0.55	0.58
	SGLT2i	46 ± 4	45 ± 3	44 ± 2	43 ± 2	44 ± 3	45 ± 4	44 ± 4	46 ± 4	44 ± 4			
	NHE-1i	42 ± 2	44 ± 2	42 ± 1	42 ± 1	42 ± 1	42 ± 1	40 ± 3	40 ± 2	42 ± 3			
Arterial PO_2 (mmHg)	Control	110 ± 11	103 ± 11	113 ± 9	101 ± 11	108 ± 9	111 ± 6	107 ± 5	110 ± 4	105 ± 5	0.01	0.23	0.21
	SGLT2i	122 ± 10	125 ± 6	129 ± 10	131 ± 8	129 ± 9	121 ± 12	115 ± 10	104 ± 8	112 ± 13			
	NHE-1i	123 ± 10	132 ± 9	133 ± 9	130 ± 9	130 ± 3	126 ± 10	127 ± 10	122 ± 8	119 ± 7			
Coronary Venous PO_2 (mmHg)	Control	20 ± 1	22 ± 2	23 ± 2	26 ± 5*	22 ± 2	20 ± 2	21 ± 2	20 ± 1	19 ± 1	0.02	0.17	0.46
	SGLT2i	18 ± 2	17 ± 1	18 ± 2	18 ± 2†	17 ± 2	17 ± 2	16 ± 2	17 ± 2	15 ± 2			
	NHE-1i	19 ± 2	20 ± 2	18 ± 2	18 ± 2†	18 ± 2	17 ± 2	17 ± 2	17 ± 2	17 ± 2			
HCT (%)	Control	31 ± 2	34 ± 3	33 ± 3	34 ± 3	32 ± 3	31 ± 2	31 ± 2	30 ± 2	32 ± 2	0.46	0.61	0.90
	SGLT2i	30 ± 1	31 ± 1	31 ± 2	31 ± 2	31 ± 2	30 ± 2	31 ± 2	31 ± 3	30 ± 3			
	NHE-1i	32 ± 2	33 ± 2	32 ± 1	34 ± 1	33 ± 1	33 ± 2	33 ± 2	33 ± 2	35 ± 2			
Arterial O_2 Content (mL/dL)	Control	15 ± 1	16 ± 1	16 ± 1	16 ± 1	15 ± 1	15 ± 1	15 ± 1	15 ± 1	15 ± 1	0.59	0.59	0.98
	SGLT2i	15 ± 1	15 ± 1	15 ± 1	15 ± 1	15 ± 1	15 ± 1	15 ± 1	15 ± 1	14 ± 1			
	NHE-1i	16 ± 1	16 ± 1	16 ± 1	16 ± 1	16 ± 1	16 ± 1	16 ± 1	16 ± 1	16 ± 1			
Coronary Venous O_2 Content (mL/dL)	Control	3.6 ± 0.5	4.4 ± 0.7	4.7 ± 0.8	5.9 ± 2.0	4.3 ± 0.7	3.5 ± 0.5	3.8 ± 0.6	3.3 ± 0.2	3.2 ± 0.3	0.08	0.43	0.41
	SGLT2i	3.5 ± 0.6	3.1 ± 0.5	3.5 ± 0.6	3.4 ± 0.6	3.3 ± 0.5	3.3 ± 0.4	3.4 ± 0.5	3.2 ± 0.5	3.0 ± 0.5			

	NHE-1i	3.7 ± 0.5	3.9 ± 1.0	3.4 ± 0.6	3.4 ± 0.4	3.2 ± 0.3	3.1 ± 0.4	3.2 ± 0.2	3.0 ± 0.2	3.1 ± 0.2			
Myocardial Oxygen Consumption (mL O2/min/g)	Control	61 ± 7	65 ± 12	53 ± 7	46 ± 6	48 ± 4	49 ± 6	48 ± 6	47 ± 7	50 ± 6	0.01	0.56	0.20
	SGLT2i	41 ± 4	45 ± 5	42 ± 3	42 ± 4	44 ± 5	38 ± 3	43 ± 7	49 ± 9	50 ± 6			
	NHE-1i	66 ± 18	71 ± 21	39 ± 2*	45 ± 5	46 ± 7	59 ± 13	54 ± 9	51 ± 9	51 ± 9			

Values are mean ± SE for Control (n = 6), SGLT2i (canagliflozin) (n = 6) and NHE-1 (cariporide) (n = 6). * $P < 0.05$ vs. baseline value (same treatment), † $P < 0.05$ vs. Control (same time point).

Table 3.2
Effects of canagliflozin and cariporide on systemic hemodynamics and cardiac contractile function

Parameter	Treatment	Condition									Condition *P*-value	Treatment *P*-value	Interaction *P*-value
		Baseline	Ischemia				Reperfusion						
		0 min	15 min	30 min	45 min	60 min	30 min	60 min	90 min	120 min			
Systolic Pressure (mmHg)	Control	115 ± 8	98 ± 7*	91 ± 7*	88 ± 4*	83 ± 5*	80 ± 2*	81 ± 4*	79 ± 4*	77 ± 6*	<0.001	0.33	0.60
	SGLT2i	110 ± 5	97 ± 9*	93 ± 9*	93 ± 10*	89 ± 9*	80 ± 7*^	67 ± 6*	70 ± 5*	80 ± 5*			
	NHE-1i	119 ± 8	108 ± 8*	100 ± 10*	99 ± 6*	100 ± 6*	93 ± 6*	92 ± 6*	84 ± 6*	83 ± 7*			
Systemic Diastolic Pressure (mmHg)	Control	77 ± 7	69 ± 7*	63 ± 5*	61 ± 4*	57 ± 5*	55 ± 2*	54 ± 4*	52 ± 4*	50 ± 5*	<0.001	0.39	0.31
	SGLT2i	81 ± 4	71 ± 6*	67 ± 7*	68 ± 8*	64 ± 7*	55 ± 5*	45 ± 4*	47 ± 3*	52 ± 3*			
	NHE-1i	81 ± 6	74 ± 5	69 ± 6	69 ± 8	69 ± 4	65 ± 5*	66 ± 5*	59 ± 5*	57 ± 5*			
Mean Pressure (mmHg)	Control	89 ± 7	79 ± 7*	72 ± 6*	70 ± 4*	66 ± 5*	64 ± 2*	63 ± 4*	61 ± 4*	60 ± 5*	<0.001	0.36	0.43
	SGLT2i	91 ± 5	80 ± 7*	76 ± 7*	77 ± 8*	73 ± 7*	63 ± 6*	52 ± 5*^	55 ± 4*	62 ± 4*			
	NHE-1i	94 ± 7	85 ± 6	79 ± 7	79 ± 9	79 ± 4	74 ± 4*	74 ± 6*	67 ± 5*	65 ± 5*			
Heart Rate (beats/min)	Control	83 ± 8	87 ± 6	83 ± 7	83 ± 5	81 ± 6	92 ± 8	91 ± 5	91 ± 5	94 ± 6	<0.001	0.01	0.02
	SGLT2i	79 ± 3	78 ± 5	73 ± 3	83 ± 5	82 ± 5	98 ± 7	113 ± 4*†^	118 ± 6*†^	113 ± 3*†^			
	NHE-1i	68 ± 6	69 ± 6	65 ± 5	74 ± 7	77 ± 10	80 ± 4	86 ± 5	83 ± 4	84 ± 3			
Systemic Vascular Resistance (mmHg*min/mL)	Control	0.03 ± 0.00	0.03 ± 0.01	0.02 ± 0.01	0.02 ± 0.00	0.02 ± 0.00	0.02 ± 0.00	0.02 ± 0.01	0.02 ± 0.00	0.02 ± 0.00	0.57	0.22	0.76
	SGLT2i	0.02 ± 0.00	0.02 ± 0.00	0.02 ± 0.00	0.01 ± 0.00	0.02 ± 0.00	0.02 ± 0.00	0.02 ± 0.00	0.02 ± 0.00	0.02 ± 0.00			
	NHE-1i	0.03 ± 0.00	0.02 ± 0.00	0.02 ± 0.00	0.02 ± 0.00	0.02 ± 0.00	0.03 ± 0.02	0.02 ± 0.00	0.02 ± 0.00	0.02 ± 0.00			
Coronary Blood Flow (µl/min/g)	Control	0.53 ± 0.06	0.57 ± 0.09	0.48 ± 0.06	0.49 ± 0.05	0.44 ± 0.05	0.44 ± 0.06	0.45 ± 0.07	0.44 ± 0.06	0.42 ± 0.06	0.005	0.55	0.07
	SGLT2i	0.37 ± 0.07	0.37 ± 0.03	0.36 ± 0.03	0.36 ± 0.04	0.37 ± 0.05	0.36 ± 0.05	0.37 ± 0.07	0.40 ± 0.06	0.44 ± 0.05			
	NHE-1i	0.57 ± 0.16	0.61 ± 0.20	0.33 ± 0.03*	0.35 ± 0.04*	0.36 ± 0.06*	0.47 ± 0.10*	0.43 ± 0.07*	0.39 ± 0.07	0.38 ± 0.06			
Ejection Fraction (%)	Control	64 ± 4	49 ± 4	48 ± 5	51 ± 3	61 ± 6	64 ± 4	63 ± 5	62 ± 5	59 ± 5	0.17	0.77	0.04
	SGLT2i	66 ± 5	64 ± 3	65 ± 3	66 ± 3	64 ± 5	62 ± 9	55 ± 7	61 ± 5	60 ± 4			

	NHE-1i	68 ± 4	50 ± 6	58 ± 7	56 ± 7	55 ± 8	61 ± 8	60 ± 7	65 ± 4	64 ± 7			
dP/dt_{max} (mmHg/s)	Control	1853 ± 138	1644 ± 301	1051 ± 214*	1365 ± 68	1325 ± 93	1408 ± 75	1339 ± 57	1623 ± 200	1661 ± 245	<0.001	0.92	0.26
	SGLT2i	1711 ± 176	1356 ± 200	1449 ± 156	1381 ± 181	1564 ± 119	1440 ± 66	1249 ± 116	1456 ± 128	1641 ± 114			
	NHE-1i	1892 ± 111	1495 ± 103	1305 ± 120*	1507 ± 114	1377 ± 150*	1335 ± 47*	1371 ± 73*	1301± 84*	1309 ± 130*			
dP/dt_{min} (mmHg/s)	Control	-1754 ± 157	-1182 ± 134*	-872 ± 192*	-1124 ± 116*	-1091 ± 123*	-1108 ± 54*	-1031 ± 118*	-1096 ± 131*	-1069 ± 147*	<0.001	0.97	0.054
	SGLT2i	-1500 ± 78	-1225 ± 129	-1191 ± 132	-1225 ± 154	-1249 ± 131	-1073 ± 105*	-900 ± 131*	-1039 ± 163*	-1238 ± 149*			
	NHE-1i	-1466 ± 106	-1221 ± 116	-1109 ± 100	-1328 ± 135	-1161 ± 85	-1048 ± 77*	-1144 ± 113	-1005 ± 81*	-982 ± 103*			
$Tau_{1/2}$ (ms)	Control	33 ± 4	45 ± 13	27 ± 6	34 ± 4	34 ± 4	38 ± 10	29 ± 2	29 ± 1	27 ± 2	<0.001	0.18	0.62
	SGLT2i	35 ± 3	35 ± 4	38 ± 5	33 ± 5	30 ± 5	26 ± 3	19 ± 4	21 ± 2	20 ± 2			
	NHE-1i	39 ± 3	51 ± 12	40 ± 4	42 ± 5	38 ± 3	32 ± 3	30 ± 3	35 ± 5	33 ± 3			
$Tau_{1/e}$ (ms)	Control	23 ± 3	31 ± 8	19 ± 4	23 ± 3	23 ± 3	27 ± 8	20 ± 2	20 ± 1	18 ± 1	<0.001	0.21	0.61
	SGLT2i	24 ± 2	24 ± 3	24 ± 3	23 ± 3	20 ± 4	17 ± 2	13 ± 2	14 ± 1	14 ± 1			
	NHE-1i	27 ± 3	32 ± 6	27 ± 3	25 ± 2	26 ± 2	22 ± 2	21 ± 2	22 ± 2	22 ± 2			

Values are mean ± SE for Control (n = 6), SGLT2i (canagliflozin) (n = 6) and NHE-1 (cariporide) (n = 6). * $P < 0.05$ vs. baseline value (same treatment), † $P < 0.05$ vs. Control (same time point).

Table 3.3
Effects of canagliflozin and cariporide on circulating substrate concentrations

Parameter	Treatment	Condition									Condition *P*-value	Treatment *P*-value	Interaction *P*-value
		Baseline	Ischemia				Reperfusion						
		0 min	15 min	30 min	45 min	60 min	30 min	60 min	90 min	120 min			
Arterial Glucose (mg/dL)	Control	114 ± 30	148 ± 40	143 ± 37	124 ± 32	139 ± 33	133 ± 36	128 ± 28	146± 32	146 ± 29	0.07	0.66	0.02
	SGLT2i	159 ± 24	151 ± 28	140 ± 22	135 ± 19	128 ± 13	135 ± 16	137 ± 18	143 ± 16	145 ± 19			
	NHE-1i	163 ± 13	184 ± 21	171 ± 17	175 ± 18	179 ± 25	153 ± 11	151 ± 20	155 ± 19	153 ± 17			
Arterial Lactate (mmol/L)	Control	1.8 ± 0.4	3.4 ± 0.9*	3.6 ± 0.9*	3.6 ± 0.9*	3.9 ± 0.9*	3.6 ± 0.8	3.4 ± 0.8*	3.2 ± 0.8*	2.7 ± 0.5	<0.001	0.46	0.97
	SGLT2i	3.5 ± 0.9	4.1 ± 1.2	4.5 ± 1.4	5.0 ± 1.2	4.6 ± 0.9	4.6 ± 1.0	4.3 ± 1.0	4.4 ± 1.2	4.0 ± 1.3			
	NHE-1i	2.6 ± 0.5	3.1 ± 0.5	3.1 ± 0.5	3.4 ± 0.7	3.5 ± 0.8	3.0 ± 0.7	2.8 ± 0.5	2.7 ± 0.5	2.5 ± 0.4			
Arterial Ketone (µmol/L))	Control	66 ± 15	94 ± 17	101 ± 21*	91 ± 19*	103 ± 21*	102 ± 18*	109 ± 16*	123 ± 19*	133 ± 24*	<0.001	0.12	0.63
	SGLT2i	57 ± 13	75 ± 19	75 ± 17	80 ± 18	80 ± 16	90 ± 17*	92 ± 16*	89 ± 11*	94 ± 12*			
	NHE-1i	35 ± 5	49 ± 7	46 ± 5	50 ± 6	58 ± 9	54 ± 6	65 ± 9*	66 ± 7*	79 ± 13*			
Arterial FFA (mmol/L)	Control	0.7 ± 0.1	0.9 ± 0.1	0.9 ± 0.1	0.8 ± 0.1	0.9 ± 0.1	0.8 ±0.1	0.8 ± 0.1	0.8 ± 0.1	0.8 ± 0.1	0.18	0.77	0.98
	SGLT2i	0.7 ± 0.1	0.7 ± 0.1	0.8 ± 0.1	0.8 ± 0.1	0.8 ± 0.1	0.8 ± 0.1	0.7 ± 0.	0.7 ± 0.1	0.6 ± 0.1			
	NHE-1i	0.8± 0.2	0.8 ± 0.2	0.8 ± 0.1	0.8 ± 0.1	0.9 ± 0.2	0.8 ± 0.1	0.8 ± 0.1	0.7 ± 0.1	0.8 ± 0.1			

Values are mean ± SE for Control (n = 6), SGLT2i (canagliflozin) (n = 6) and NHE-1 (cariporide) (n=6). * $P < 0.05$ vs. baseline value (same treatment), † $P < 0.05$ vs. Control (same time point).

Chapter 4: Discussion

Summary of Findings

Cardiovascular disease is a major health concern worldwide. Contributing to the cardiovascular burden is the continued epidemic of obesity and the increased prevalence of type 2 diabetes. The combination of obesity and type 2 diabetes are associated with several complicated and distinct cardiovascular pathophysiological changes. Treating dysmetabolic patients has become problematic as several anti-hyperglycemic agents have shown worsening or no improvements on cardiovascular outcomes. Recent cardiovascular outcome trials evaluating the SGLT2i class of anti-hyperglycemic agents has demonstrated surprising and unexpected improvements in cardiovascular outcomes. SGLT2i were designed to reduce blood glucose levels by inhibiting glucose reabsorption in kidney by inhibiting the SGLT2 in the proximal tubules. SGLT2i were never expected to have cardioprotective effects especially since SGLT2 protein expression has not been detected in the human heart. Therefore, the mechanism behind their cardiovascular action remains a mystery.

There have been several proposed hypotheses for the SGLT2i mediated cardioprotective effects, but uncertainty remains. The goal of this application was to test the two main hypotheses for the observed effects in the acute ischemia reperfusion porcine model, first the “thrifty fuel” hypothesis and second the “sodium” hypothesis. SGLT2i therapy has been demonstrated to augment circulating ketone bodies via alterations in hepatic metabolism [48, 112]. This “thrifty fuel” effect could serve to shift myocardial substrate utilization away from free fatty acids toward ketones, which require less oxygen per mole of ATP produced [22]. Alternatively, recent evidence also suggests that the SGLT2i mediate cardiac protection via direct inhibition of the sodium hydrogen exchanger-1 (NHE-1), i.e. “the sodium hypothesis” [19, 149]. In conditions such as

myocardial ischemia, intracellular acidosis activates NHE-1 leading to myocyte sodium accumulation and calcium overload. Inhibition of NHE-1 during myocardial ischemia could potentially improve calcium handling and preserve cardiomyocyte function and efficiency.

Although prior studies have demonstrated improved cardiac function with increased ketones [58, 77, 115] or the administration of an NHE-1i [53, 67, 142], a direct link to SGLT2i mediated improvements in cardiac function via increased ketones or inhibition of NHE-1 has not been established. Accordingly, the goal of this investigation was to test the hypothesis that SGLT2i therapy enhances cardiac function and mitigates myocardial infarct size via indirect effects on NHE-1 and/or a shift in myocardial substrate utilization. The findings of the Specific Aims are summarized below:

Aim 1: Test the hypothesis that SGLT2i improves cardiac contractile function and efficiency and attenuates infarct size in response to ischemia/reperfusion injury via salutary effects on myocardial metabolism.

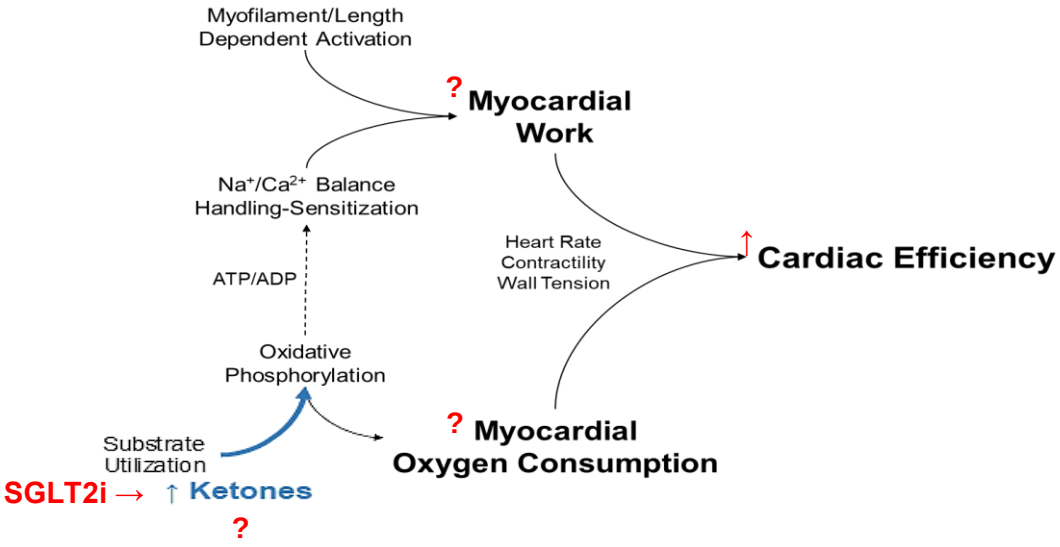


Figure 4.1 Schematic diagram of the proposed ketone effects of SGLT2i on the primary determinants of cardiac efficiency.

The goal of the initial study was to evaluate the effects of SGLT2i on cardiac contractile function, substrate utilization, and efficiency before and during regional myocardial ischemia/reperfusion injury in normal, metabolically healthy swine (Figure 4.1). This study was designed to evaluate short-term effects of SGLT2i (24 hour exposure) thereby excluding contributions of effects of chronic SGLT2i exposure (i.e. weight loss, reduced blood pressure, etc.). The initial study revealed that treatment with SGLT2i produced significant increases in end diastolic volume at the onset of ischemia and that this increased volume returned to baseline values upon reperfusion (Figure 2.3). This canagliflozin mediated increase in end diastolic volume was directly associated with increased stroke volume and stroke work relative to controls during ischemia (Figure 2.5). Taken together, the increased stroke work and unaltered myocardial oxygen consumption (Table 2.1) led to improved cardiac work efficiency during ischemia in the SGLT2i treated group compared to the control group. Additionally, no differences in myocardial uptake of glucose, lactate, free fatty acids or ketones, were noted between treatment groups at any time (Figure 2.6).

Additional studies were designed to evaluate the effect of SGLT2i on infarct size in response to ischemia/reperfusion injury. These studies used a longer (60 minute) coronary occlusion and a 2-hour reperfusion period. Canagliflozin increased both end diastolic volume and stroke volume as observed in the initial studies (Appendix B). Canagliflozin treatment also produced significant reductions in infarct size relative to control animals (Figure 2.7). These data demonstrate that SGLT2i with canagliflozin preserves cardiac contractile function and efficiency during regional myocardial ischemia and provides ischemia protection independent of alterations in myocardial substrate utilization. Collectively, these observations argue against contributions of ketone-induced shifts in fuel selection (“thrifty fuel hypothesis”) to the cardioprotective effects of SGLT2i in normal, metabolically healthy swine (Figure 4.2).

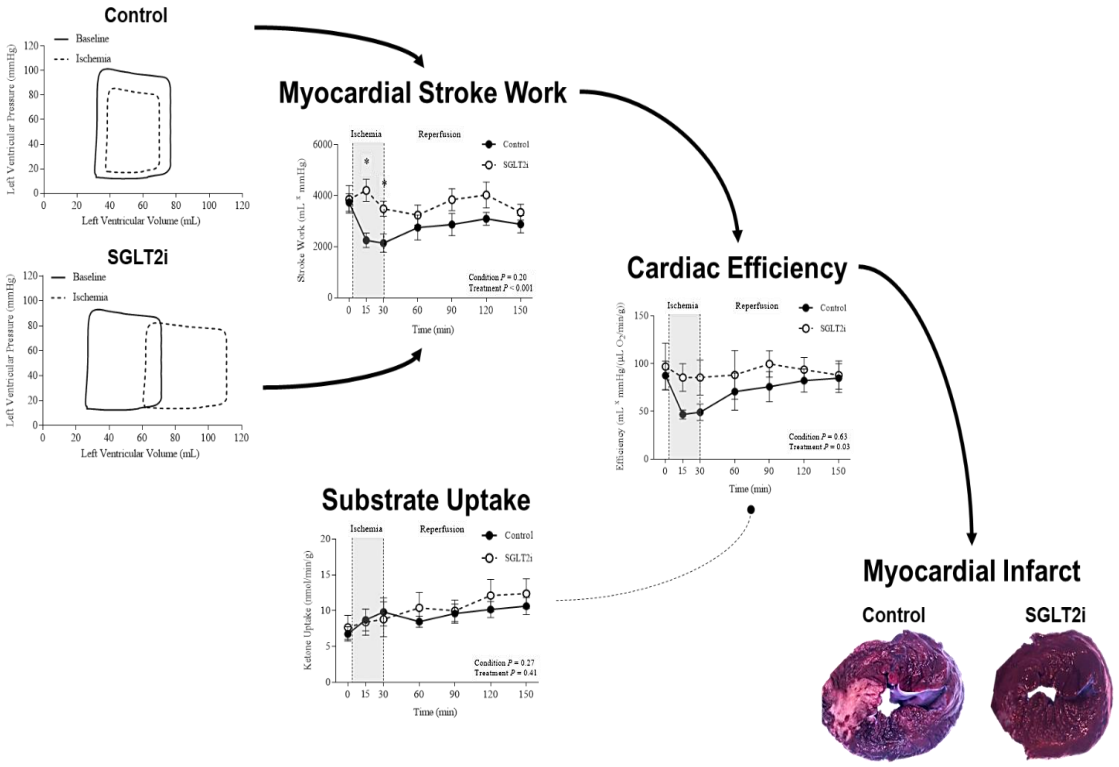


Figure 4.2 Schematic diagram of the experimental outcomes of SGLT2i effects on the primary determinants of cardiac efficiency.

SGLT2i could preserve cardiac contractile function during regional myocardial ischemia via activation of the Frank-Starling mechanism, increase in cardiac output via an increase in end diastolic volume as was demonstrated in Aim 1 (Figure 2.4 C and D). Studies have shown the giant protein titin contributes to this mechanism which brings us to the question could SGLT2i alter titin's contribution to the Frank-Starling mechanism [5, 52, 93]. Titin functions as a "molecular spring" and is the primary source of "passive" tension in cardiomyocytes (sarcomeres) [62]. Additionally, studies have demonstrated that changes in titin, (i.e. differences titin isoforms N2B and N2BA, increases in N2B cause the heart to become more rigid) and PTM of titin N2B and N2BA isoforms have been observed during ischemia/reperfusion contributing to contractile dysfunction in the heart [8, 27, 91].

Altered function of cardiac ion channels, transporters and/or exchangers are often the consequence of an ischemic event which can lead to improper Ca^{2+} handling [21, 30]. Dysfunctional Ca^{2+} handling is detrimental to the mechanical and electrical properties of the heart (diastolic and systolic dysfunction, arrhythmias) [30, 120]. It has been suggested that SGLT2i could play a role in Ca^{2+} handling in cardiomyocytes. Baartscheer and Uthman have suggested that SGLT2i inhibits NHE-1 activity thereby preventing increased intracellular Na^{+} and consequentially intracellular Ca^{2+} overload during ischemia and that this is how SGLT2i improve cardiovascular outcomes during ischemia [19, 149]. This hypothesis is discussed and addressed in Aim 2 of this investigation. Additionally, Lee et al [94] suggests empagliflozin attenuates Na^{+} and Ca^{2+} dysregulation in streptozotocin-induced diabetic rats, a model of diabetes mellitus (DM) cardiomyopathy. They concluded that empagliflozin significantly altered Ca^{2+} regulation, late Na^{+} and NHE currents and electrophysical properties of DM cardiomyopathy, therefore proposing this as the mechanism for the reported SGLT2i cardioprotection [94]. While Lee et al. demonstrated improvements in Na^{+} and Ca^{2+} handling in DM cardiomyopathy, the question for whether SGLT2i influence Na^{+} and Ca^{2+} handling in normal glycemic hearts during ischemia remains.

Aim 2: Evaluate the hypothesis that NHE-1 contributes to the cardiac effects of SGLT2i in response to myocardial ischemia/reperfusion injury.

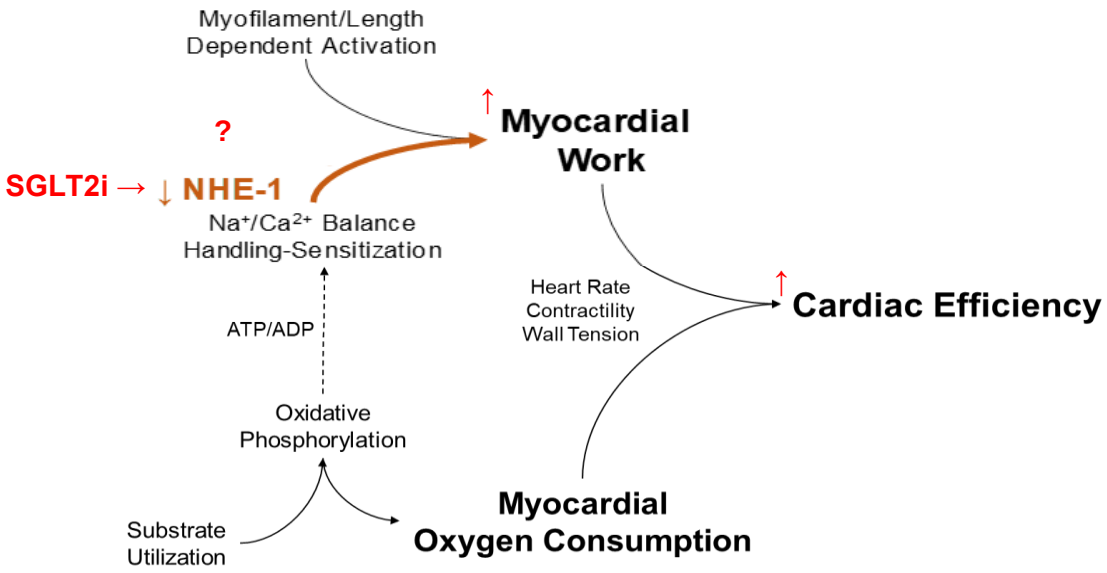


Figure 4.3 Schematic diagram of the proposed NHE-1 effects of SGLT2i on the primary determinants of cardiac efficiency.

The goal of Aim 2 was to evaluate the effects of SGLT2i and NHE-1i on cardiac contractile function and efficiency (Figure 4.3) in response to a regional myocardial ischemia/reperfusion injury in normal, metabolically healthy swine. Additionally, this study was designed to evaluate acute effects of SGLT2i (15-30 min, I.V. infusion) thereby excluding contributions of additional pleiotropic effects beyond those that are directly affecting the heart (i.e. weight loss, reduced blood pressure, etc.). Our initial study revealed that acute treatment with SGLT2i produced significant increases in end diastolic volume at the onset of ischemia and that this increased volume returned to baseline values upon reperfusion (Figure 3.1). This canagliflozin mediated increase in end diastolic volume was directly associated with increased stroke work and improved cardiac

efficiency compared to controls during ischemia (Figure 3.3). In contrast to canagliflozin, cariporide did not alter ventricular filling volume, cardiac output or work efficiency at any time point during the study period.

To further examine effects of SGLT2i on NHE-1 a cellular approach was utilized (in parallel to the *in vivo* studies). The initial NHE-1 activity studies evaluated SGLT2i (canagliflozin and dapagliflozin) in WT human NHE-1 in transfected CHO (AP-1) cells. This allowed evaluation of whether SGLT2i had a direct effect on NHE-1 activity. The data from the WT human NHE-1 in transfected CHO (AP-1) cell studies failed to show an effect of SGLT2i on NHE-1 activity, specifically SGLT2i did not inhibit NHE-1 in response to an acid load (similar to the acidic conditions in cardiomyocytes during ischemia). It has been proposed that the SGTL2i mediated cardiovascular protection was at least in part due to SGLT2i inhibition of NHE-1 activity ("sodium hypothesis") however, findings from these studies do not support this hypothesis (Figure 4.4).

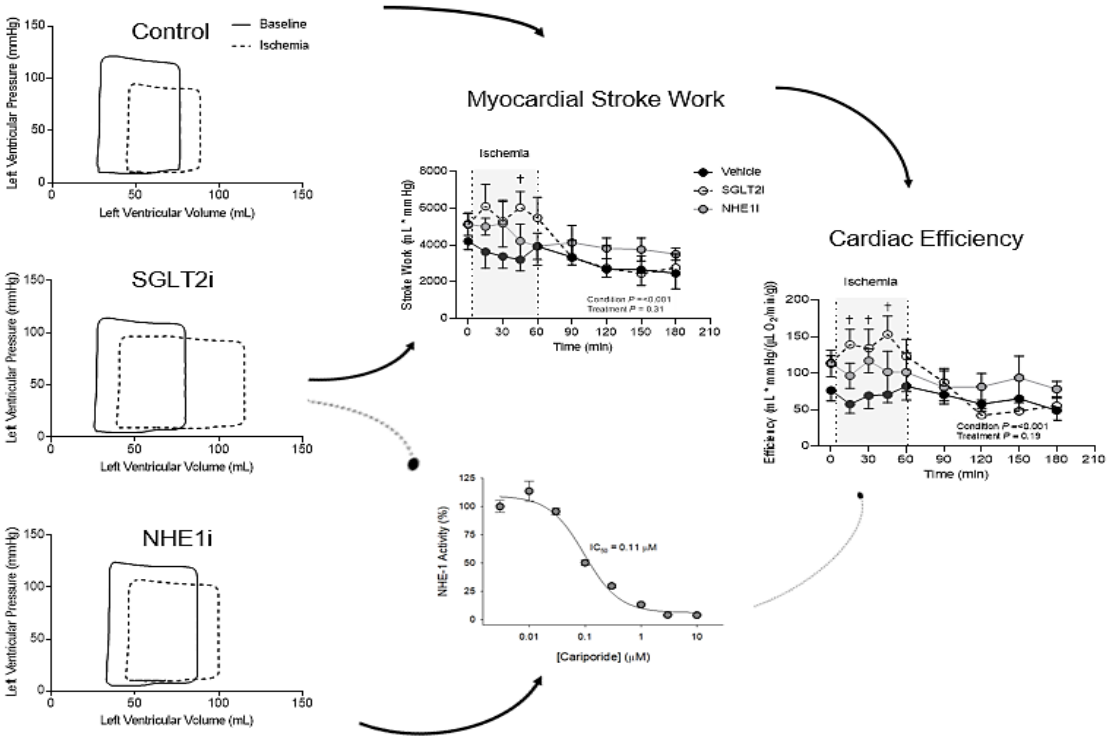


Figure 4.4 Schematic diagram of the experimental outcomes of the proposed NHE1 effects of SGLT2i on the primary determinants of cardiac efficiency.

It has been reported that both canagliflozin and cariporide have in infarct mitigating effects [20, 89, 131]. Nevertheless, canagliflozin and cariporide had very different cardiac contractile and efficiency responses to ischemia. This would suggest that SGLT2i and NHE-1i are working through different mechanisms (i.e. SGLT2i does not affect NHE-1 activity). It has also been reported that the infarct mitigating effects of cariporide are only present when dosed prior to or at the time of ischemia but the effect is lost if dosed after the onset of ischemia suggesting a localized effect (i.e. NHE-1i needs to be present within the area at risk prior to the ischemic event) [89]. Whereas, in addition to infarct mitigation properties, canagliflozin also demonstrated a more "global" cardiac response to ischemia (i.e. increased cardiac filling volumes, stroke work and cardiac output). Studies published by Sayour et. al provide further support for a more "global" response with SGLT2i as they demonstrated cardiac effects and infarct mitigation when canagliflozin was administered

5 minutes into ischemia [131]. Together these data suggest that SGLT2i have a cardiac effect that exists outside of the ischemic territory but is triggered by an ischemic event. These data further suggest SGLT2i could be mediating the activity of a cardiac ion channel, exchanger or some other mechanism directly involved in the myocardium's immediate response to ischemia.

Implications

Findings from this investigation provides support that the SGLT2i canagliflozin preserves cardiac contractile function and efficiency during regional myocardial ischemia and provides ischemia protection independent of alterations in myocardial substrate utilization or NHE-1 activity. The findings from these studies demonstrate an acute action of SGLT2i on cardiac function at the onset of ischemia and for the duration of the ischemic period. Canagliflozin had no effect on cardiac function at baseline and returned to baseline levels at the beginning of the reperfusion. These data suggest SGLT2i could be mediating the activity of a cardiac ion channel, exchanger or some other mechanism directly involved in the myocardium's immediate response to ischemia. The observed cardiac effects of SGLT2i in the initial study are consistent with the activation of a Frank-Starling mechanism. The Frank-Starling law states that as cardiac filling increases (left ventricular end diastolic volume) there is a proportionate increase in stroke volume [40]. Despite over 100 years of research, the precise mechanism responsible for this fundamental physiologic response remains a contested mystery. Recent studies indicate that the giant protein titin, a major component of the contractile apparatus, contributes to the activation of the Frank-Starling mechanism [99] by acting as an adjustable "molecular spring" which allows the heart to quickly balance ventricular stroke volume relative to diastolic filling volume on a relative beat-to-beat basis [62, 91, 113]. In particular, titin phosphorylation has been observed during ischemia/reperfusion and in heart failure

contributing to contractile dysfunction in the heart [62, 66, 78, 126]. A recent study reported direct effects of empagliflozin on cardiac muscle resulting in improved diastolic function in human and mouse heart failure myocardium [118]. The improved diastolic function was suggested to be the result of empagliflozin mediated increases in titin phosphorylation. Additionally, these data were evident after acute (15-60 minute) empagliflozin treatment and were independent of any changes in systolic contractility or cytosolic Ca^{2+}. Together these data support a potential role for SGLT2i in modulating titin phosphorylation which could explain the observed cardiac effects of the present study and warrant further investigation. Additional findings support that that SGLT2i attenuates myocardial infarct size (Figure 2.7). The infarct mitigating properties of canagliflozin suggest SGLT2i plays a role in myocardial survival during ischemia/reperfusion (i.e. STAT3, SAFE pathway, mitochondrial transition pore) [12]. While there are many proposed mechanisms for the observed SGLT2i mediated effects on cardiac function there are few answers. The findings from these studies begin to answer some those questions thereby allowing us to identify and better understand the mechanisms behind the SGLT2i mediated cardioprotection. Additionally, the findings of this investigation further support a role for SGLT2i use in non-diabetic individuals at risk for ischemic heart disease.

Future Directions and Proposed Studies

While these studies answer mechanistic questions regarding SGLT2i role in cardioprotection during myocardial ischemia/reperfusion injury in a metabolically normal animal, the question remains as to whether this holds true in the setting of diabetes where metabolic flexibility is often compromised. Therefore, future studies are needed to evaluate cardiac effects of SGLT2i during ischemia/reperfusion in obese diabetic animals as well as metabolically normal and diabetic heart failure models. With the reported

improvements in heart failure outcomes in the SGLT2i clinical trials, we also propose that future studies should include evaluating the hypothesis that SGLT2i attenuates the progression of heart failure in the setting of obesity.

Unfortunately, relevant large animal models representing diabetic heart failure are currently lacking. However, our lab has completed preliminary heart failure model development studies in Ossabaw swine. Ossabaw swine, when fed a high fat/ high cholesterol diet become obese, develop insulin resistance/impaired glucose tolerance, dyslipidemia and atherosclerosis have a similar phenotype as to what is observed in "metabolic syndrome" (MetS). We have established that rapid ventricular pacing (~180 beats/min for ~4-5 weeks) of obese Ossabaw swine induces marked increases in left ventricular end diastolic pressure (> 20 mmHg), moderate increases in end diastolic and end systolic volume, but without changes in ejection fraction; i.e. mimicking the clinical phenotype of heart failure with preserved ejection fraction (HFpEF) (Figure 4.5).

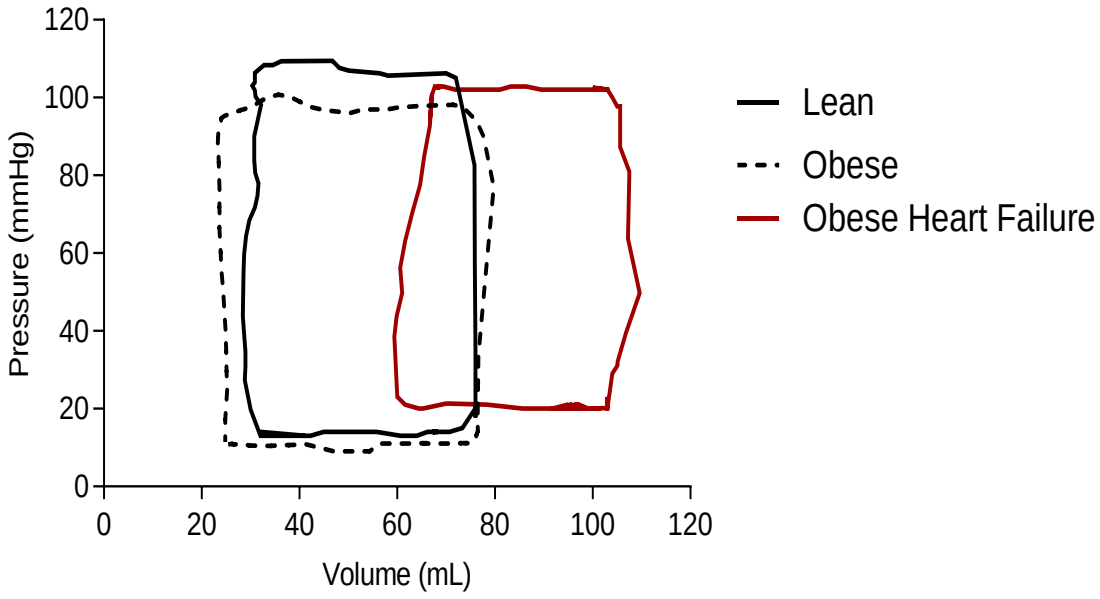


Figure 4.5 Representative pressure-volume loops for lean, obese and obese heart failure Ossabaw swine.

This obese pacing-induced heart failure model now gives us the opportunity to specifically evaluate the distinct physiologic, pathophysiologic and metabolic changes associated with this dysmetabolic state (metabolomic and proteomic analysis, histology to evaluate structural changes, hemodynamic analysis including chronic telemetric assessment of left ventricular pressure, volume, coronary blood flow, arterial pressure at rest and during interventions such as graded treadmill exercise, ischemia-reperfusion injury). This model will allow us to perform studies to evaluate the effects of SGLT2i on cardiac function in the setting of obese heart failure.

It has been reported that SGLT2 expression is not present in the human heart [32, 43, 153] and, data from our lab have demonstrated similar results in pig hearts (Figure 2.1). The expression data would indicate that SGLT2i should not have a direct effect on the heart as SGLT2 protein expression isn't present. However, the data generated by our lab demonstrate cardiac effects with acute administration (15 minute intravenous infusion, immediately prior to ischemia) and raise the possibility that SGLT2i are having a direct effect on the heart. This would indicate that SGLT2i has off-target molecular effects either during ischemia. Therefore, we propose studies to investigate molecular targets that SGLT2i could potentially be interacting with in the myocardium in the presence/absence of ischemia. These studies would be a collaborative effort and involve a chemoproteomics approach (via click chemistry) to identify proteins from myocardial lysates and cardiac myocytes under normal and ischemic conditions that bind to SGLT2i.

Click chemistry describes the bioconjugation reaction between an azide with an alkyne group to form a covalent bond under a Cu(I) catalyzed condition (Figure 4.6) [160]. The overall process is as follows:

1. Canagliflozin and a structurally similar but SGLT2 inactive compound will be incubated separately with cells (either under normal or hypoxic conditions) and/or porcine myocardial lysates.

2. Samples will be exposed to UV light to crosslink canagliflozin compounds to protein binding targets.
3. Biotin-azide or biotin-alkyne and CuAAC will be added to initiate the click reaction.
4. Tagged proteins will be purified using streptavidin beads.
5. Protein identification will be done by mass spectrometry proteomics.
6. The negative control compound will be used to eliminate non-specific binding proteins.
7. Samples with free canagliflozin compound will be spiked into additional samples to serve as an additional negative control.

This analysis could potentially identify additional novel protein ligands of SGLT2i that may lead to new pathways for cardiac protection.

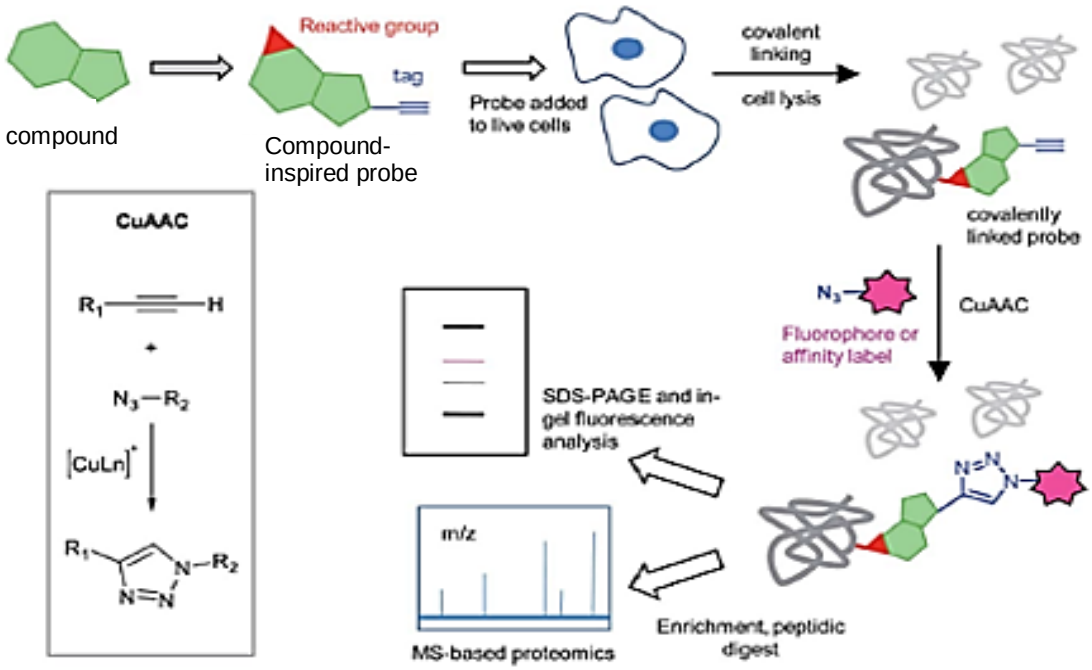


Figure 4.6 Schematic diagram of the proposed chemical proteomics study to identify binding partners/targets. [160]

Concluding Remarks

Obesity and type 2 diabetes are highly prevalent diseases that are significant contributors to the increasing cardiovascular disease burden. Furthermore, the combination of obesity and type 2 diabetes is associated with increased prevalence and severity of cardiovascular disease. To date many type 2 diabetes therapies have either failed to reduce adverse cardiovascular outcomes or have been associated with worsening of heart disease. Together this highlights the need for new therapeutic options for treating cardiovascular disease in dysmetabolic individuals. Recent clinical trials EMPA-REG, CANVAS, DECLARE-TIMI, demonstrated that empagliflozin, canagliflozin and dapagliflozin improved cardiovascular outcomes in patients with type 2 diabetes. These SGLT2 inhibitors are a new class of anti-hyperglycemic agent, which produced unexpected reductions of sudden cardiac death and heart failure hospitalizations. While these inhibitors provide a valuable treatment option for dysmetabolic individuals with cardiovascular disease, little is known regarding their cardioprotective mechanism of action. The goal of this work was to investigate the two main hypotheses for the observed effects, the “thrifty fuel” hypothesis and the “sodium” hypothesis in the setting of acute cardiac ischemia and reperfusion. Findings from this investigation provides support that the SGLT2i canagliflozin preserves cardiac contractile function and efficiency during regional myocardial ischemia and provides ischemia protection independent of alterations in myocardial substrate utilization or NHE-1 activity. The findings from these studies demonstrate an acute action of SGLT2i on cardiac function at the onset of ischemia and for the duration of the ischemic period. While there are still many questions regarding SGLT2i cardioprotective mechanisms, this investigation has provided valuable information for two of the most prevalent hypotheses. Furthermore, the acute nature of SGLT2i mediated cardiac effects identified in this body of work emphasizes the need to focus on mechanisms directly associated with the myocardium (i.e. cardiac ion channels,

exchangers, mitochondrial preservation). Additionally, the findings of this investigation further support a role for SGLT2i use in non-diabetic individuals at risk for ischemic heart disease.

Appendices

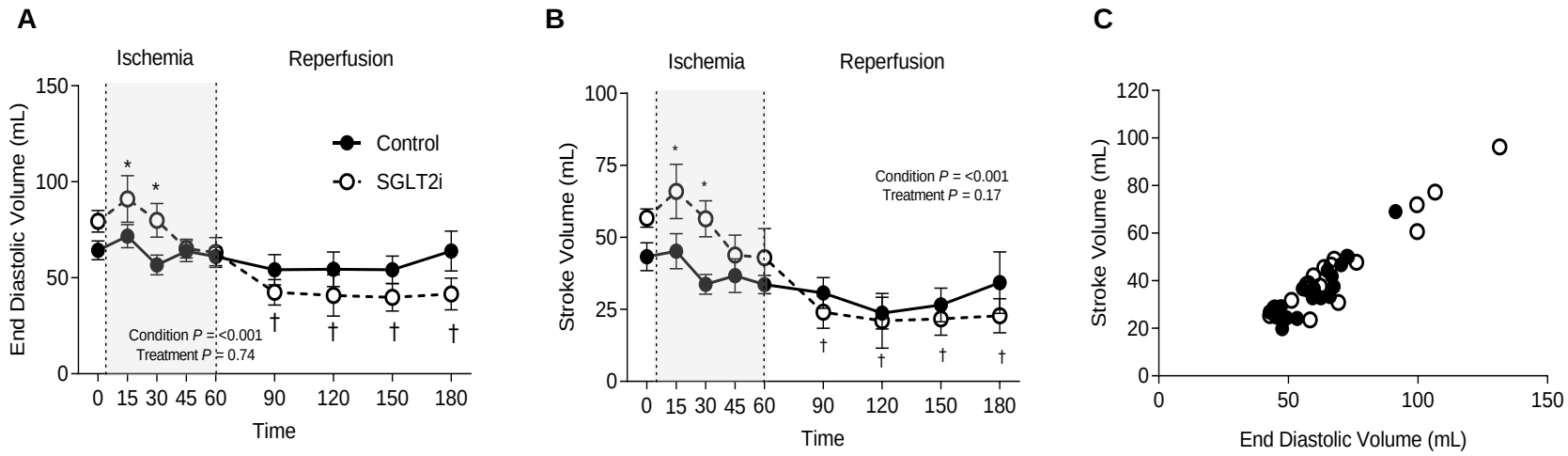
Appendix A: Supplemental Table

Effects of canagliflozin on systemic hemodynamics and blood gas parameters

Parameter	Treatment	Condition									Condition *P*-value	Treatment *P*-value	Interaction *P*-value
		Baseline	Ischemia				Reperfusion						
		0 min	15 min	30 min	45 min	60 min	90 min	120 min	150 min	180 min			
Arterial pH	Control	7.53 ± 0.58	7.52 ± 0.06	7.53 ± 0.06	7.51 ± 0.04	7.49 ± 0.03	7.49 ± 0.03	7.50 ± 0.04	7.50 ± 0.05	7.49 ± 0.05	0.815	0.899	0.908
	SGLT2i	7.51 ± 0.07	7.50 ± 0.06	7.51 ± 0.05	7.51 ± 0.04	7.51 ± 0.04	7.51 ± 0.05	7.51 ± 0.04	7.48 ± 0.04	7.51 ± 0.05			
Arterial PCO2 (mmHg)	Control	38 ± 5	38 ± 5	37 ± 5	40 ± 3	40 ± 3	40 ± 3	39 ± 4	39 ± 5	39 ± 5	0.995	0.004	0.800
	SGLT2i	43 ± 7 *	43 ± 6 *	42 ± 6	42 ± 3	41 ± 3	40 ± 4	40 ± 3	41 ± 4	40 ± 3			
Arterial PO_2 (mmHg)	Control	169 ± 24	168 ± 23	168 ± 29	164 ± 37	169 ± 23	171 ± 19	165 ± 26	165 ± 25	161 ± 26	0.988	0.468	0.998
	SGLT2i	166 ± 32	168 ± 31	171 ± 29	166 ± 32	165 ± 32	156 ± 32	160 ± 31	153 ± 40	156 ± 37			
HCT (%)	Control	33 ± 4	33 ± 3	33 ± 4	34 ± 3	35 ± 3	34 ± 2	34 ± 2	33 ± 5	33 ± 3	0.993	0.592	0.942
	SGLT2i	33 ± 3	34 ± 4	34 ± 4	34 ± 4	34 ± 3	33 ± 3	33 ± 3	35 ± 2	35 ± 4			
Mean Blood Pressure (mmHg)	Control	75 ± 11	75 ± 8	70 ± 12	73 ± 13	73 ± 14	70 ± 16	65 ± 19	63 ± 20	65 ± 14	0.272	0.470	0.999
	SGLT2i	76 ± 17	75 ± 14	73 ± 16	68 ± 18	70 ± 16	64 ± 17	61 ± 14	60 ± 15	62 ± 14			
Heart Rate (beats/minute)	Control	83 ± 21	78 ± 18	85 ± 13	91 ± 10	88 ± 13	86 ± 14	85 ± 12	87 ± 10	82 ± 7	0.483	<0.001	0.262
	SGLT2i	67 ± 20	66 ± 14	62 ±16 *	61 ± 15 *	60 ± 13 *	82 ± 24	82 ± 30	76 ± 22	82 ± 7			

Values are mean ± SE for saline (n = 6) and canagliflozin (n = 6). *p<0.05 vs. time control. [23]

Appendix B: Supplemental Figure



Effect of ischemia/reperfusion injury on end diastolic volume (Panel A), stroke volume (Panel B) and the relationship between stroke volume and end diastolic volume during ischemia in Control (n = 5) and SGLT2i (canagliflozin) treated (n = 5) swine. * $P < 0.05$ vs. Control (same time point), † $P < 0.05$ vs. baseline value (same treatment). [23]

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CURRICULUM VITAE

Hana Elisabeth Baker

EDUCATION

Indiana University 2020
PhD, Cellular & Integrative Physiology

Indiana Institute of Technology Indianapolis 2004
MBA, Management and Human Resources

Purdue University West Lafayette 1998
BS, Health Science

RESEARCH EXPERIENCE

Johnathan D. Tune, PhD Laboratory 2017– present
Cellular & Integrative Physiology, IU School of Medicine

PROFESSIONAL EXPERIENCE

Eli Lilly and Company 2009 - present
Consultant Biologist

- Drug discovery scientist with extensive experience in pharmacology, in vivo biology, small molecule and biomolecule discovery and development. Scientific expertise in dyslipidemia, cardiovascular disease, diabetes and diabetic nephropathy. Recruiting industry professionals to attend bi-annual networking events

HONORS AND AWARDS

Young Investigator Award 2018
Society for Experimental Biology and Medicine

Research Excellence Award 2019
IU Center for Diabetes & Metabolic Diseases 2019 Annual Symposium

PUBLICATIONS

1. Tune JD, Baker HE, Berwick ZC, Moberly SP, Casalini ED, Noblet JN, Zhen E, Kowala MC, Christe ME, Goodwill AG. Cardiac and coronary effects of (Pyr)Apelin-13 are mediated by alterations in peripheral resistance. *Am J Physiol Heart Circ Physiol. In review*

2. Tune JD, Goodwill AG, Kiel AM, Baker HE, Bender SB, Merkus D, Duncker DJ. Disentangling the Gordian knot of local metabolic control of coronary blood flow. *Am J Physiol Heart Circ Physiol. In Press.* PMID 31702972

3. Baker HE, Tune JD, Goodwill AG, Mather KJ. Benefits of empagliflozin beyond enhancing myocardial energetics? *J Am Coll Cardiol.* 74:825-826, 2019. PMID 31395139

4. Baker HE, Kiel AM, Luebbe ST, Simon BR, Earl CC, Regmi A, Roell WC, Mather KJ, Tune JD, Goodwill AG. Inhibition of sodium-glucose cotransporter-2 preserves cardiac function during regional myocardial ischemia independent of alterations in myocardial substrate utilization. Basic Res Cardiol. 2019 Apr 19;114(3):25. doi: 10.1007/s00395-019-0733-2. PubMed PMID: 31004234.

5. Kiel AM, Goodwill AG, Baker HE, Dick GM, Tune JD. Local metabolic hypothesis is not sufficient to explain coronary autoregulatory behavior. Basic Res Cardiol. 2018 Aug 2;113(5):33. doi: 10.1007/s00395-018-0691-0. PubMed PMID: 30073416.

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8. Okragly AJ, Hamang MJ, Pena EA, Baker HE, Bullock HA, Lucchesi J, Martin AP, Ma YL, Benschop RJ. Elevated levels of Interleukin (IL)-33 induce bone pathology but absence of IL-33 does not negatively impact normal bone homeostasis. Cytokine 79: 66-73, 2016.

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11. Shah U, Boyle CD, Chackalamannil S, Baker H, Kowalski T, Lee J, Terracina G, Zhang L. Azabicyclic sulfonamides as potent 11beta-HSD1 inhibitors. Bioorg Med Chem Lett. 20(5): 1551-4, 2010.

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13. Terracina G, Lee J, Stamford A, Lankin C, Neelamkavil S, Shah U, Boyle C, Baker H, Kowalski T, Zhang X, Sorota S, Parker E and Zhang L. Identification of three potential chemical lead series for 11-β-Hydroxysterol Dehydrogenase type 1 (11β-HSD1) inhibitors as potential treatments for metabolic syndrome. American Society for Neuroscience, Abstract. Washington D.C., November 12-16, 2005

14. Graham JM, Baker H, Nisenbaum LK and Hemrick-Luecke SK. Comparison of the effects of intracisternal administration of Orexin A and Orexin B on rat serum corticosterone and ACTH concentrations. American Society for Neuroscience, Abstract. San Diego, CA, October 23-27, 2004

POSTER/ORAL PRESENTATIONS

1. Indiana Physiological Society Meeting 2017, Oral presentation. Preclinical rodent models demonstrate TG lowering but no other metabolic effects following ANGPTL3 neutralization.

2. IU Center for Diabetes & Metabolic Diseases 2017 Annual Symposium, Hana E. Baker, Melissa A. Bellinger, Lan Yu, and Laura F. Michael Preclinical rodent models demonstrate triglyceride lowering but no other metabolic effects following ANGPTL3 neutralization.

3. Indiana Physiological Society Meeting 2018, Oral presentation. Inhibition of Sodium Glucose Cotransporter-2 Preserves Cardiac Function during Regional Myocardial Ischemia via a Frank-Starling Mechanism.

4. Experimental Biology 2018, Hana E Baker, Alexander M Kiel, Kieren J Mather, Johnathan D Tune, Adam G Goodwill. Inhibition of Sodium Glucose Cotransporter-2 Preserves Cardiac Function during Regional Myocardial Ischemia via a Frank-Starling Mechanism.

5. IU Center for Diabetes & Metabolic Diseases 2019 Annual Symposium, Hana E Baker, Adam G Goodwill, Kieren J Mather, Mark C Kowala, Johnathan D Tune. Inhibition of Sodium Glucose Cotransporter-2 Mitigates Heart Failure Progression in Obesity.

INVITED LECTURES

- Innovations across Lilly, Grand Rounds, Eli Lilly and Company, Indianapolis, In August 2, 2017